nature portfolio

Peer Review File

MeCP2 gene dosage-dependent neurodevelopmentally restricted defects arise by aberrant activation of cell fate-determining bivalent genes

Corresponding Author: Dr Vania Broccoli

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 manuscript NCOMMS-25-42033-T entitled “MeCP2 gene dosage-dependent neurodevelopmentally restricted defects arise by SWI/SNF-mediated aberrant activation of cell fate-determining bivalent genes” by Luoni and colleagues addresses the cell type-specific consequences of increased MECP2 gene dosage—a question of ongoing relevance in both neurodevelopmental disease research and gene therapy development. The authors compare neural progenitor cells (NPCs) and mature neurons to assess how MeCP2 overexpression affects transcriptional and functional outputs in these two cellular contexts. The study aims to provide mechanistic insight into why MeCP2 dosage sensitivity appears to vary between early developmental stages and the mature brain.
The authors report that Mecp2 overexpression causes significant transcriptional and phenotypic alterations in NPCs, but only minimal effects in neurons. These differences are attributed to distinct chromatin binding dynamics and endogenous Mecp2 levels, with a proposed role for the SWI/SNF chromatin remodeling complex in mediating the effects in progenitor cells. Based on these findings, the authors argue that Mecp2 overexpression may be better tolerated in the mature brain, with implications for the safety of gene therapy approaches in Rett syndrome.
The study shows a clear and well-structured presentation of the data, especially the figures showing side-by-side comparisons of NPCs and neurons. These small panel overviews are highly informative and make it easy to follow which experimental conditions and cell types are being analyzed.
While the study addresses an important question and presents a series of intriguing observations, several aspects of the experimental design and mechanistic interpretation raise concerns that should be carefully addressed before publication.
1) In the first paragraph of the results section, the authors state that different multiplicities of infection (MOIs) were tested, resulting in the use of an MOI of 10 for NPCs and 5 for mouse neurons. However, no data are shown to support this titration process. Furthermore, for human iPSC-derived neurons, an MOI of 10 was used, differing from the MOI applied to mouse neurons, without explanation. The rationale behind these dosing decisions remains unclear. To properly assess the comparability of Mecp2 overexpression across cell types, the authors should provide supporting titration data—e.g. in a supplementary figure—and clarify the reasoning behind the chosen MOIs. This would also help ensure that the observed differences between NPCs and neurons are not simply due to disparities in expression levels.
2) In the in vivo experiments, in utero electroporation was used to overexpress Mecp2, and the authors mention that the same lentiviral plasmid as in the in vitro experiments was employed. However, they also state that EGFP was co-expressed with Mecp2 in the electroporated cells. This raises questions about the actual vector design used for electroporation. Was a different plasmid construct used for these experiments? If so, this should be clearly stated. Additionally, details about the vector design—such as whether a P2A or IRES sequence was used to co-express EGFP and Mecp2—are missing and should be provided to clarify how expression of both proteins was achieved in vivo.
3) In Supplementary Figure 3, the authors show that cells electroporated with Mecp2 + GFP display increased expression levels. However, it is not specified in the Materials and Methods section under which imaging conditions the brain sections were acquired. Were the same microscope settings (e.g., laser power, gain, exposure time) used for all conditions? The Mecp2-overexpressing cells appear somewhat overexposed, which raises concerns about the comparability of signal intensity. These details should be provided to ensure that the observed differences in expression are not due to imaging artifacts.
4) When examining Figure 7, it is striking that the baseline mean frequency of spontaneous EPSCs in control animals treated with the AAV-PHP.eB vector appears substantially higher than in control animals subjected to in utero

electroporation. The same is true for the amplitude, which is already increased in the AAV-PHP.eB control group compared to the in utero electroporated controls. This raises concerns about the comparability of the two experimental systems and calls into question whether the observed effects on EPSC frequency can truly be attributed to Mecp2 overexpression, rather than to differences between the delivery methods or developmental timing. The authors should clarify this point and discuss whether these baseline differences might confound the interpretation of Mecp2's effects on synaptic function.
5) This also raises the question of why recordings were performed from pyramidal neurons in cortical layer 2/3. Given that in utero electroporation was carried out at embryonic day 13.5, one would expect predominantly deeper layer neurons—such as those in layer 5—to be transfected. It is therefore unclear why layer 2/3 neurons were selected for electrophysiological recordings. The authors should clarify the rationale for choosing this cell population and explain how this aligns with the timing and targeting of the electroporation.
6) Given that the study also includes a human system using iPSC-derived neurons, it is unclear why neural progenitor cells were not examined in the human context. Since the key findings in the mouse model highlight strong effects of Mecp2 overexpression specifically in NPCs, it would have been valuable to include human NPCs to confirm whether similar mechanisms are at play. The absence of this parallel limits the extent to which the mouse findings can be directly translated to the human system. The authors should comment on this limitation and explain why human NPCs were not included in the study.
7) Several important methodological details remain unclear in the Materials and Methods section, which are essential for evaluating the reproducibility and interpretation of the experiments. First, it is not specified which wild-type mouse strain was used for the in vitro and in vivo experiments, nor whether the same strain was used consistently across all experiments. The cited reference (Zaghi et al., 2023) does not describe the isolation or culturing of murine NPCs, but rather focuses on human cells. A more appropriate citation or a detailed description of the murine NPC culture protocol should be provided. Additionally, for all cell-based experiments, key parameters such as the number of cells seeded per condition, whether cells were grown on coverslips, and specifics of the transduction or transfection setup are missing. For the in utero electroporation, the orientation or angle of the electrodes should also be specified, as this directly influences the targeting of cortical layers. Figure legends also lack important information—for example, in Figure 7 and Supplementary Figure 3, the number of recorded cells should be provided alongside the number of animals. Moreover, in Supplementary Figure 3, the scale bar cannot plausibly represent 200 µm in both panels A and B; this should be corrected. Finally, several figure panels (e.g., Figures 2E and 3B) contain font sizes that are too small to be easily legible and should be improved for clarity.
8) On page 12, at the beginning of the paragraph, the authors state: "Consistent with previous studies in MDS mouse models...", but no citation is provided to support this statement. A reference should be added here to acknowledge the relevant prior work and to clarify which studies the authors are referring to.
9) In Figure 3B, the panel showing the fraction of CpG islands fully covered by Mecp2 would benefit from a visual marker—such as an arrow or highlight—to draw attention to this specific data point. This would help emphasize the key finding and improve clarity for the reader.

Reviewer #2

(Remarks to the Author)
Luoni et al address an important question of whether MECP2 over-expression in NPC or neurons is detrimental to their function, which has major implications for gene therapy for Rett syndrome. They use both mouse and human stem cell models, and show that V5Tagged MECP2 over-expression in NPCs leads to profound transcriptional changes accompanied by SWI/SNF dependent chromatin remodelling at CpG islands. In contrast, minimal changes at most were observed in neurons, the V5Tagged MECP2 protein was more rapidly degraded and was less accessible to bind to CpG islands that were prebound by the high levels of endogenous MECP2. They conclude that MECP2 over-expression is expected to be well tolerated in gene therapy for RTT.

Overall, this a high quality study that uses new approaches including 4f-SAMMY-Seq, with accurate interpretations of the molecular differences between MECP2-endo and MECP2-exo in the two cell types. It is appropriate for publication in Nature Communications after addressing minor concerns.

Comments

1. This reviewer was initially very concerned that the MECP2-exo transgene was not shown to rescue the RTT phenotype in this manuscript, until reading Ref 53 which is cited for AAV and iPSC differentiation methods. Please cite Luoni et al 2020 much earlier in the text to document that the V5Tagged MECP2-exo is functional after AAV transfer and rescues RTT behavior phenotypes but does not affect WT mice. This is important to validate the gene therapy conclusions of the current manuscript. It might also be useful to state that the instability properties of the MECP2-exo transgene make it an excellent probe for molecular studies on chromatin accessibility.

If over-expression only has an effect in NPCs, does this have implications for MDS gene therapy? Does it suggest that MDS correction in neurons will not correct functional changes that were established in NPCs at an earlier developmental timepoint?

2. The CHX protein experiments do not appear to rule out that it is the N term V5 tag that reduces MECP2-exo stability in neurons, and technically they can be improved. Are they also different from the findings previously reported in Ref 53 showing no difference in protein stability in neurons? In Fig 6C-D, why do the Mecp2 levels decrease in LV:GFP NPCs but trend to an increase in Ns – has the Ns panel been accidentally flipped horizontally? Since MECP2-endo does not decline in the LV:GFP Ns, it suggests that CHX treatment of the Ns sample is less complete than in NPCs. A more convincing

experiment would be to do more CHX timepoints and calculate the protein half-life in NPCs and Ns. This would also clarify whether the MECP2-endo has the same or different half-life in NPCs and Ns, which could impact the model that MECP2-exo protein stability is driven by chromatin accessibility differences.

3. Why do Figs 6A-B not show the 600 mM lanes that are quantified below? These are more important than the 800 mM lanes that are barely detectable and not significantly different.

4. The resolution of gene names in Fig 5E is very fuzzy. The gene name should be added to Fig 3I top panel.

5. References 20-23 in the Intro do not refer to AAV gene therapy. These references are likely intended to be Refs 68-73 that are used in the Discussion.

Reviewer #3

(Remarks to the Author)
In this manuscript, Luoni et al. sought to investigate the molecular and phenotypic consequences of acute increased MECP2 dosage introduced in either neuronal progenitors (NPCs) or in neurons (Ns). This study contrasts with the existing body of literature, which has studied the consequences of chronic increased MECP2 dosage in either the germline of mice or from genomic duplication in human cells. The authors tested the hypothesis that differentiated neurons are more tolerant to an acute increase in MECP2 dosage than NPCs. To test this hypothesis, the authors cultured NPCs or Ns from the embryonic mouse and used lentivirus to exogenously express human MECP2. Importantly, the authors took care to titrate the viral dose to identify conditions that allowed for modest and disease-relevant MeCP2 overexpression. With the optimized system, the authors performed a series of transcriptomics and epigenomic approaches to understand the molecular consequences and differences in each respective cell type. They first found that MECP2 overexpression drives significantly more and higher magnitude gene expression changes in NPCs than Ns. Using CUT&TAG, the authors distinguish binding profiles of the endogenous MeCP2 and exogenous MeCP2 and find that while the global binding patterns are similar, in NPCs there was a greater deposition of exogenous MeCP2 to CpG islands compared to N. Interestingly, in NPCs, a large proportion of this enrichment of exogenous MeCP2 resided in bivalently poised genes, whose expression is mediated by Smarc1b. Exogenous MeCP2 did not drastically alter the global chromatin state within a cell type, though the overall landscape is different between NPCs and Ns. Additionally, in Ns, exogenous MeCP2 displayed a reduced affinity to DNA and prone to degradation, suggesting the high baseline expression of MeCP2 in Ns is “saturating” on DNA and protecting from exogenous MeCP2. Lastly, the authors test the functional consequences of post-conception MeCP2 overexpression by either starting overexpression embryonically or in an adult mouse. They strikingly find abnormal electrophysiology only when MeCP2 was expressed early on, suggesting early increased dosage of MeCP2 is detrimental to neuronal function, but that the adult brain/neuron is more tolerant to increased MeCP2 dosage. The core concept of the data were corroborated in human neurons, where acute overexpression of MeCP2 led to a very modest change in gene expression. Overall, this is an interesting study exploring a potential understudied role of MeCP2 dosage in neural progenitors, with implications towards therapies in MeCP2-related disorders. However, the overall message that neurons are tolerant of MeCP2 dosage is overstated given the data presented and the body of literature.

Major comments:

1. While the number of differentially expressed genes and their magnitude are no doubt different between the NPCs and Ns, some additional context is needed to interpret the Ns differential gene expression. Given that cultured neurons are quite immature and tend to have low levels of non-CG methylation, and that non-CG methylation (particularly mCA) has a strong influence on MeCP2-dependent gene regulation (Gabel et al. Nature 2015), what is the non-CG methylation status (and differences) between NPCs and Ns? For example, if the cultured Ns have no mCH (H = ACT), how relevant would the lessons learned from this study bear on mature neurons in vivo. Bisulfite sequencing would help provide clarity on these questions and help to determine if known trends with mCH and MeCP2 are observed in vitro between NPCs and Ns.
2. The MeCP2 CUT&TAG data, measuring both endogenous and exogenous MeCP2 binding, is elegantly designed. However, the localization of MeCP2 to CpG islands is only partially the story. Given the recent exciting evidence that MeCP2 binds in hotspots and non-methylated DNA, it would be important to analyze the data generated in this study to investigate if non-methyl binding or binding in intragenic regions is different between endogenous and exogenous MeCP2 and if that binding pattern is different between NPCs and Ns.
3. The notion of subtle gene expression changes from acute MeCP2 increases in neurons has been observed before (Ito Ishida et al. J Neurosci. 2021). In Ito Ishida et al, the authors used focal AAV mediated MECP2 overexpression postnatally and performed behavior and transcriptomics. The authors should compare the transcriptomic signature generated in this study (Figure 12, see “low”, “medium”, “high” overexpression in DEG number) to the profile generated in neurons in this study. It would be important to dissect the similarities or differences of acute MECP2 dosage on neurons that matured in vitro versus in vivo. Furthermore, despite the subtle molecular changes observed in the “low” MeCP2 overexpression population, Ito-Ishida et al. still observed behavioral deficits overall due to MeCP2 overexpression (Figure 11). Though the electrophysiology in this study is strong, it would be important and helpful to benchmark the behavioral consequence of IUE vs AAV-PHP.eB-MECP2 overexpression. The authors should perform a standard battery of tests to assess to what extent (if at all) IUE MECP2 or AAV-PHP.eB-MECP2 overexpression phenocopies MECP2 duplication mice.
4. The results in Figure 6 are intriguing but incomplete. Its not overtly clear why excess MeCP2 should have differential affinity or targeted for degradation given that MeCP2 is not static on DNA (Piccolo et al. eLife 2019). The authors should test if post-translational modifications (e.g., phosphorylation) on the exogenous MeCP2 differentiate the endogenous and exogenous “isoforms”. Even if negative, these data would be powerful in determining if exogenous MeCP2 behaves the

same biochemically as endogenous MeCP2 in the protein-protein networks (beyond DNA binding as wonderfully measured in this paper already).
5. MeCP2 protein levels have a had a lagging effect on gene expression in vivo and in vivo (Bajikar et al. Neuron 2025, Shao et al. Sci Transl Med 2021, Bajikar et al. Human Mol Genet 2025). 5 days between viral transduction and downstream RNA changes is on the same time scale as the “earliest” timepoints where changes were observed in these studies. Additionally, MeCP2 levels rise during neuronal maturation (Shao et al. Genes Dev 2021, Bajikar et al. Human Mol Genet 2025). The authors could expound on the context of their study by assessing MeCP2 levels overtime with their culture protocol and assessing the transcriptome at a later time point (e.g., 4 weeks) after infection. One could hypothesize the N transcriptome will progressively dysregulate matching in vivo expectations, whereas the NPCs could be more stable or adapt (or indeed still remain more susceptible to increased MeCP2 dosage!).
6. The phrase “no effects” in mature neurons is overstated, as transcriptional dysregulation is not the only disrupted output in Mecp2 mutant neurons. There are many phenotypes that were not assessed in vitro or in vivo (e.g., morphology, circuit level disruptions etc). More caution is needed throughout the manuscript, unless these non-molecular phenotypic experiments are performed.

Minor comments:

1. Please provide the differential gene expression statistics/output for all detected genes and denote the direction of fold change to provide as a complete resource for the field.
2. Please provide a blot of the NPC and N MeCP2 levels on the same blot for visual/quantitative comparison. This was somewhat gleaned from the CUT&TAG data, but the range of the displays was not always readable.
3. The methods section is incomplete, particularly for the bioinformatics section. Package software versions are left as placeholders.
4. Please ensure the scale and labels for all genome browser tracks are legible.
5. The axis labels for Figure 6A,B do not agree with the labels on the blot. Please put the correct values as appropriate.
6. The citations on line 91 for studies that used “supraphysiological viral doses” does not appear to correspond to the appropriate references at the end. These must be critically fixed for readers to find these papers easily.
7. There is a typo on line 372, “MSD” should be MDS

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I have carefully reviewed the authors’ point-by-point responses as well as the updated text and figures. All of my previous concerns have been addressed to my satisfaction. Overall, the revisions have strengthened the manuscript considerably. In particular, the added methodological details and clarifications substantially improve the transparency and reproducibility of the work, and the revised Materials and Methods section is now much clearer and easier to follow.
Before final acceptance, I would ask the authors to carefully proofread the manuscript once more for consistency and clarity. Specifically, please verify that the correction of the in utero electroporation timing from E13.5 to E14.5 has been implemented consistently throughout the text, figure legends, and especially in Figure 7.

Reviewer #2

(Remarks to the Author)
The authors have addressed all the concerns raised by this reviewer. The new Fig 6D shows equivalent half-life for the Mecp2-Exo-v5 tag in both cell types as expected.

Reviewer #3

(Remarks to the Author)
The authors have addressed my concerns by performing additional experiments and analysis to strengthen their conclusions, while also appropriately tempering interpretations where needed.

I have a minor suggestion to improve the manuscript: the labels on several graph axes, legends, or scales are too small and not readily legible especially on a printout (e.g., Figure 1c, Figure 2c-e, Figure 3b,c,i, Figure 4f-j, Figure 5e-f, Figure 7d). The authors could consider resizing the labels on these graphs.

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Title: MeCP2 gene dosage-dependent neurodevelopmentally restricted defects arise by SWI/SNF-mediated aberrant activation of cell fate-determining bivalent genes

Authors: Mirko Luoni, Michal Kubacki, Serena Gea Giannelli, Francesca Morosi, Claudia Di Berardino, Angelo Iannielli, Alessandro Sessa, Gaia Colasante, Emmanuele Di Patrizio Soldateschi, Chiara Lanzuolo, Vania Broccoli

Point-by-point letter

Reviewer #1

The manuscript NCOMMS-25-42033-T entitled "MeCP2 gene dosage-dependent neurodevelopmentally restricted defects arise by SWI/SNF-mediated aberrant activation of cell fate-determining bivalent genes" by Luoni and colleagues addresses the cell type-specific consequences of increased MECP2 gene dosage—a question of ongoing relevance in both neurodevelopmental disease research and gene therapy development. The authors compare neural progenitor cells (NPCs) and mature neurons to assess how MeCP2 overexpression affects transcriptional and functional outputs in these two cellular contexts. The study aims to provide mechanistic insight into why MeCP2 dosage sensitivity appears to vary between early developmental stages and the mature brain. The authors report that Mecp2 overexpression causes significant transcriptional and phenotypic alterations in NPCs, but only minimal effects in neurons. These differences are attributed to distinct chromatin binding dynamics and endogenous Mecp2 levels, with a proposed role for the SWI/SNF chromatin remodeling complex in mediating the effects in progenitor cells. Based on these findings, the authors argue that Mecp2 overexpression may be better tolerated in the mature brain, with implications for the safety of gene therapy approaches in Rett syndrome.
The study shows a clear and well-structured presentation of the data, especially the figures showing side-by-side comparisons of NPCs and neurons. These small panel overviews are highly informative and make it easy to follow which experimental conditions and cell types are being analyzed.
While the study addresses an important question and presents a series of intriguing observations, several aspects of the experimental design and mechanistic interpretation raise concerns that should be carefully addressed before publication.

1) In the first paragraph of the results section, the authors state that different multiplicities of infection (MOIs) were tested, resulting in the use of an MOI of 10 for NPCs and 5 for mouse neurons. However, no data are shown to support this titration process. Furthermore, for human iPSC-derived neurons, an MOI of 10 was used, differing from the MOI applied to mouse neurons, without explanation. The rationale behind these dosing decisions remains unclear. To properly assess the comparability of Mecp2 overexpression across cell types, the authors should provide supporting titration data—e.g. in a supplementary figure—and clarify the reasoning behind the chosen MOIs. This would also help ensure that the observed differences between NPCs and neurons are not simply due to disparities in expression levels.

Answer:
The reviewer's point is well taken. We have now included in the Supplementary Figures 1 and 5, immunostaining and western blot analyses supporting the selection of the MOIs used in each cell type, as well as for the molecular characterizations. In addition, the corresponding text in the Results section has been revised to provide a clearer explanation of the rationale behind these choices.

2) In the in vivo experiments, in utero electroporation was used to overexpress Mecp2, and the authors mention that the same lentiviral plasmid as in the in vitro experiments was employed. However, they also state that EGFP was co-expressed with Mecp2 in the electroporated cells. This raises questions about the actual vector design used for electroporation. Was a different plasmid construct used for these experiments? If so, this should be clearly stated. Additionally, details about the vector design—such as whether a P2A or IRES sequence was used to co-express EGFP and Mecp2—are missing and should be provided to clarify how expression of both proteins was achieved in vivo.

Answer:
We apologize for the lack of clarity that led to this misunderstanding. For the *in utero* electroporation (IUE) experiments, we did not use a modified Mecp2 overexpressing plasmid with EGFP fused via a P2A, IRES, or similar sequence. Instead, we co-electroporated two separate plasmids: one expressing Mecp2 (the same construct used in the other experiments) and one expressing EGFP. This is a commonly used strategy in IUE experiments, as the efficiency of co-electroporation is typically very high, resulting in most transfected cells receiving both plasmids (PMID: 11784059). We have clarified this point in the revised manuscript, specifically in the relevant Methods and Results sections.

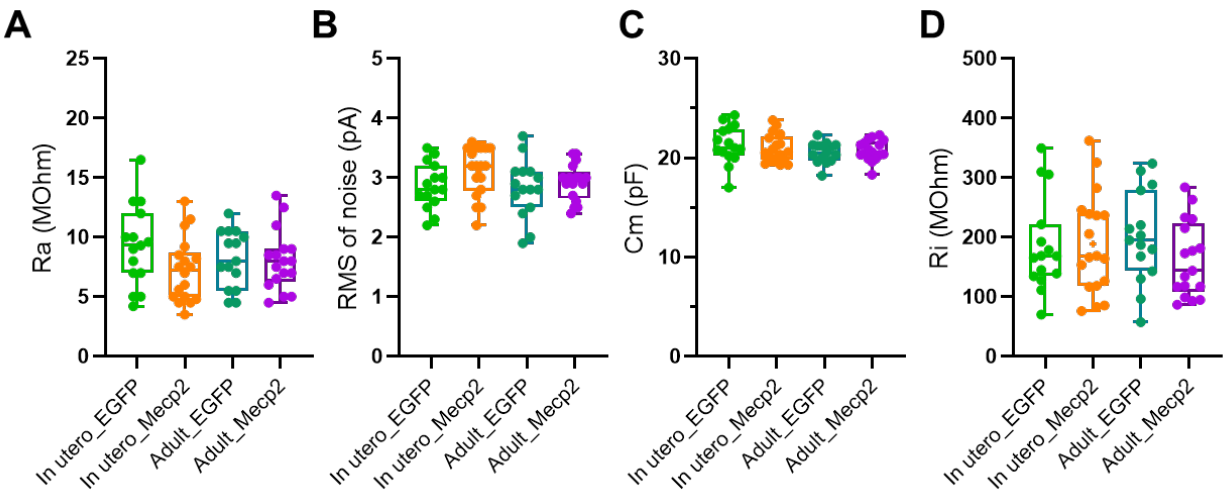
3) In Supplementary Figure 3, the authors show that cells electroporated with Mecp2 + GFP display increased expression levels. However, it is not specified in the Materials and Methods section under which imaging conditions the brain sections were acquired. Were the same microscope settings (e.g., laser power, gain, exposure time) used for all conditions? The Mecp2-overexpressing cells appear somewhat overexposed, which raises concerns about the comparability of signal intensity. These details should be provided to ensure that the observed differences in expression are not due to imaging artifacts.

Answer:
We thank the reviewer for bringing this important point to our attention. In these experiments, electroporated cells were tracked based on EGFP expression, not Mecp2. Importantly, the amount of EGFP plasmid was identical in both control (EGFP alone) and Mecp2-electroporated conditions, ensuring comparability. Based on EGFP fluorescence, we quantified the proportion of electroporated cells located in the SVZ/VZ and in the CP.
All images were acquired using identical microscope settings (laser power, gain, and exposure time), as now detailed in the revised Materials and Methods “Immunofluorescence” section.

4) When examining Figure 7, it is striking that the baseline mean frequency of spontaneous EPSCs in control animals treated with the AAV-PHP.eB vector appears substantially higher than in control animals subjected to in utero electroporation. The same is true for the amplitude, which is already increased in the AAV-PHP.eB control group compared to the in utero electroporated controls. This raises concerns about the comparability of the two experimental systems and calls into question whether the observed effects on EPSC frequency can truly be attributed to Mecp2 overexpression, rather than to differences between the delivery methods or developmental timing. The authors should clarify this point and discuss whether these baseline differences might confound the interpretation of Mecp2’s effects on synaptic function.

Answer:

We agree with the reviewer's observation that the baseline differences between the two experimental systems should be acknowledged. These differences are likely due to a technical bias: in the IUE group, a longer delay occurred between slice preparation and recording, as additional time was needed to visually locate the electroporated region and identify the best area for patching. This may have led to a partial deterioration of synaptic activity, thereby affecting the amplitude and frequency of spontaneous EPSCs in control cells.
However, it is important to note that our goal was not to directly compare AAV- (P30) and IUE (E14.5)-treated animals, but rather to assess the effect of Mecp2 overexpression within each experimental system, always comparing Mecp2-expressing cells to their respective EGFP controls.
Moreover, as shown in the figures below, there is no excessive heterogeneity in the basic recording parameters within each group or among mice, indicating the absence of significant outliers.



A-D) Box plots showing data distribution across individual mice for each of the four experimental groups (In utero-EGFP, In utero-Mecp2, Adult-EGFP, and Adult-Mecp2) for (**A**) access resistance (Ra; an indicator of recording pipette access to the intracellular space), (**B**) root mean square (RMS) noise (a measure of baseline noise used to set the event detection threshold), (**C**) membrane capacitance (Cm), and (**D**) input resistance (Ri) — two passive membrane properties that indirectly reflect neuronal size and morphology, and for Ri also the expression profile of leakage channels. All values followed a normal distribution as determined by the D'Agostino–Pearson test. None of the analyzed parameters significantly differed among groups, as assessed by one-way ANOVA, allowing us to exclude any effect of recording quality (**A–B**) or cell heterogeneity (**C–D**).

5) This also raises the question of why recordings were performed from pyramidal neurons in cortical layer 2/3. Given that in utero electroporation was carried out at embryonic day 13.5, one would expect predominantly deeper layer neurons—such as those in layer 5—to be transfected. It is therefore unclear why layer 2/3 neurons were selected for electrophysiological recordings. The authors should clarify the rationale for choosing this cell population and explain how this aligns with the timing and targeting of the electroporation.

Answer:
The reviewer is correct, and we apologize for the confusion. In the original text, we mistakenly indicated E13.5 instead of E14.5 as the timing of the *in utero* electroporation. At E14.5, we consistently observed a predominant distribution of transfected neurons in cortical layers 2/3 rather than in layer 5, and we have corrected this in the revised manuscript.

6) Given that the study also includes a human system using iPSC-derived neurons, it is unclear why neural progenitor cells were not examined in the human context. Since the key findings in the mouse model highlight strong effects of Mecp2 overexpression specifically in NPCs, it would have been valuable to include human NPCs to confirm whether similar mechanisms are at play. The absence of this parallel limits the extent to which the mouse findings can be directly translated to the human system. The authors should comment on this limitation and explain why human NPCs were not included in the study.

Answer:
The reviewer's point is well taken. We have now included the RNA-seq analysis of human NPCs in Figure 7D-E, which shows results which nicely align with those obtained in mouse NPCs. These data provide a strong confirmation about the remarkable impact of MeCP2 overexpression in this cellular model, thus supporting the translational relevance of our findings.

7) Several important methodological details remain unclear in the Materials and Methods section, which are essential for evaluating the reproducibility and interpretation of the experiments. First, it is not specified which wild-type mouse strain was used for the in vitro and in vivo experiments, nor whether the same strain was used consistently across all experiments. The cited reference (Zaghi et al., 2023) does not describe the isolation or culturing of murine NPCs, but rather focuses on human cells. A more appropriate citation or a detailed description of the murine NPC culture protocol should be provided. Additionally, for all cell-based experiments, key parameters such as the number of cells seeded per condition, whether cells were grown on coverslips, and specifics of the transduction or transfection setup are missing. For the in utero electroporation, the orientation or angle of the electrodes should also be specified, as this directly influences the targeting of cortical layers. Figure legends also lack important information—for example, in Figure 7 and Supplementary Figure 3, the number of recorded cells should be provided alongside the number of animals. Moreover, in Supplementary Figure 3, the scale bar cannot plausibly represent 200 µm in both panels A and B; this should be corrected. Finally, several figure panels (e.g., Figures 2E and 3B) contain font sizes that are too small to be easily legible and should be improved for clarity.

Answer:
All the missing or unclear methodological details have now been corrected and clarified throughout the Materials and Methods section, figure legends, and Supplementary Information. We also revised the figures to improve clarity and readability, including font sizes and scale bars, and provided additional details on mouse strain, cell culture procedures, and electroporation settings to ensure full reproducibility.

8) On page 12, at the beginning of the paragraph, the authors state: "Consistent with previous studies in MDS mouse models...", but no citation is provided to support this statement. A reference should be added here to acknowledge the relevant prior work and to clarify which studies the authors are referring to.

Answer:
We have added the appropriate references to support this statement and to acknowledge the relevant prior studies conducted in MDS mouse models.

9) In Figure 3B, the panel showing the fraction of CpG islands fully covered by Mecp2 would benefit

from a visual marker—such as an arrow or highlight—to draw attention to this specific data point. This would help emphasize the key finding and improve clarity for the reader.

Answer:
As suggested by the reviewer, we have highlighted the peak corresponding to 100% coverage in Figure 3B to make this key feature more easily visible and improve overall clarity.

Reviewer #2

Luoni et al address an important question of whether MECP2 over-expression in NPC or neurons is detrimental to their function, which has major implications for gene therapy for Rett syndrome. They use both mouse and human stem cell models, and show that V5Tagged MECP2 over-expression in NPCs leads to profound transcriptional changes accompanied by SWI/SNF dependent chromatin remodelling at CpG islands. In contrast, minimal changes at most were observed in neurons, the V5Tagged MECP2 protein was more rapidly degraded and was less accessible to bind to CpG islands that were prebound by the high levels of endogenous MECP2. They conclude that MECP2 over-expression is expected to be well tolerated in gene therapy for RTT.

Overall, this a high quality study that uses new approaches including 4f-SAMMY-Seq, with accurate interpretations of the molecular differences between MECP2-endo and MECP2-exo in the two cell types. It is appropriate for publication in Nature Communications after addressing minor concerns.

Comments

1. This reviewer was initially very concerned that the MECP2-exo transgene was not shown to rescue the RTT phenotype in this manuscript, until reading Ref 53 which is cited for AAV and iPSC differentiation methods. Please cite Luoni et al 2020 much earlier in the text to document that the V5Tagged MECP2-exo is functional after AAV transfer and rescues RTT behavior phenotypes but does not affect WT mice. This is important to validate the gene therapy conclusions of the current manuscript. It might also be useful to state that the instability properties of the MECP2-exo transgene make it an excellent probe for molecular studies on chromatin accessibility.

Answer:
We fully agree with this reviewer's comment. We have revised the text accordingly, moving the citation to Luoni et al., 2020 earlier in the manuscript to document that the V5-tagged MECP2-exo transgene is functional after AAV delivery and rescues RTT behavioral phenotypes without affecting WT mice. We have also included a note highlighting that the intrinsic instability of the MECP2-exo transgene makes it a valuable probe for studying chromatin accessibility.

If over-expression only has an effect in NPCs, does this have implications for MDS gene therapy? Does it suggest that MDS correction in neurons will not correct functional changes that were established in NPCs at an earlier developmental timepoint?

Answer:
This is indeed an excellent point. Our study suggests that the neuropathology of MDS is largely driven by developmental alterations, with neurons maturing prematurely. However, this does not necessarily imply that restoring MeCP2 levels in mature MDS neurons would be ineffective. Indeed, correcting MeCP2 expression at later stages may still lead to functional improvements, although it is unlikely to

fully rescue all deficits, as many of them originate during development. It is also important to note that current mouse models do not completely recapitulate all aspects of MDS pathology, which limits the interpretability of reversal studies. We have revised the Discussion accordingly to better reflect these considerations.

2. The CHX protein experiments do not appear to rule out that it is the N term V5 tag that reduces MECP2-exo stability in neurons, and technically they can be improved. Are they also different from the findings previously reported in Ref 53 showing no difference in protein stability in neurons?

Answer:
Regarding the previous report (Ex Ref. 53, Luoni et al., 2020), that study compared WT and Mecp2-KO neurons transduced with the iMecp2 cassette. It is therefore plausible that the stability of exogenous Mecp2 may differ in the KO background, which we did not test in the present work.
Nevertheless, the reviewer's point is well taken: although the V5 tag is small, it may still influence protein stability. To address this concern, we repeated the protein stability experiments using an alternative tag (FLAG), as shown in Figure 6C-D. In addition, we included an intermediate time point (4 hours after CHX treatment) to provide a more complete characterization (Figure 6C-D).

In Fig 6C-D, why do the Mecp2 levels decrease in LV:GFP NPCs but trend to an increase in Ns – has the Ns panel been accidentally flipped horizontally? Since MECP2-endo does not decline in the LV:GFP Ns, it suggests that CHX treatment of the Ns sample is less complete than in NPCs. A more convincing experiment would be to do more CHX timepoints and calculate the protein half-life in NPCs and Ns. This would also clarify whether the MECP2-endo has the same or different half-life in NPCs and Ns, which could impact the model that MECP2-exo protein stability is driven by chromatin accessibility differences.

We confirm that, unfortunately, the gel in the original figure had been accidentally flipped horizontally. As mentioned above, we have now corrected this error and included an additional intermediate time point (4 hours of CHX treatment) to better assess Mecp2 stability and provide a clearer and more complete analysis (Figure 6C-D).

3. Why do Figs 6A-B not show the 600 mM lanes that are quantified below? These are more important than the 800 mM lanes that are barely detectable and not significantly different.

Answer:
We are grateful to the reviewer for identifying this oversight. Indeed, the figure presented an error: the 0 mM condition was incorrectly indicated and the actual conditions range from 200 to 800 mM. We have now corrected the figure accordingly.

4. The resolution of gene names in Fig 5E is very fuzzy. The gene name should be added to Fig 3I top panel.

Answer:
We have revised both figures, eliminating the gene names which are unnecessary in Figure 5E and increasing the lettering of the gene name to the top panel of Figure 3I.

5. References 20-23 in the Intro do not refer to AAV gene therapy. These references are likely intended to be Refs 68-73 that are used in the Discussion.

Answer:
We apologize for this oversight and have revised the text accordingly, replacing the incorrect references with the appropriate ones (Refs. 68–73) related to AAV gene therapy.

Reviewer #3

In this manuscript, Luoni et al. sought to investigate the molecular and phenotypic consequences of acute increased MECP2 dosage introduced in either neuronal progenitors (NPCs) or in neurons (Ns). This study contrasts with the existing body of literature, which has studied the consequences of chronic increased MECP2 dosage in either the germline of mice or from genomic duplication in human cells. The authors tested the hypothesis that differentiated neurons are more tolerant to an acute increase in MECP2 dosage than NPCs. To test this hypothesis, the authors cultured NPCs or Ns from the embryonic mouse and used lentivirus to exogenously express human MECP2. Importantly, the authors took care to titrate the viral dose to identify conditions that allowed for modest and disease-relevant MeCP2 overexpression. With the optimized system, the authors performed a series of transcriptomics and epigenomic approaches to understand the molecular consequences and differences in each respective cell type. They first found that MECP2 overexpression drives significantly more and higher magnitude gene expression changes in NPCs than Ns. Using CUT&TAG, the authors distinguish binding profiles of the endogenous MeCP2 and exogenous MeCP2 and find that while the global binding patterns are similar, in NPCs there was a greater deposition of exogenous MeCP2 to CpG islands compared to N. Interestingly, in NPCs, a large proportion of this enrichment of exogenous MeCP2 resided in bivalently poised genes, whose expression is mediated by Smarc1b. Exogenous MeCP2 did not drastically alter the global chromatin state within a cell type, though the overall landscape is different between NPCs and Ns. Additionally, in Ns, exogenous MeCP2 displayed a reduced affinity to DNA and prone to degradation, suggesting the high baseline expression of MeCP2 in Ns is “saturating” on DNA and protecting from exogenous MeCP2. Lastly, the authors test the functional consequences of post-conception MeCP2 overexpression by either starting overexpression embryonically or in an adult mouse. They strikingly find abnormal electrophysiology only when MeCP2 was expressed early on, suggesting early increased dosage of MeCP2 is detrimental to neuronal function, but that the adult brain/neuron is more tolerant to increased MeCP2 dosage. The core concept of the data were corroborated in human neurons, where acute overexpression of MeCP2 led to a very modest change in gene expression. Overall, this is an interesting study exploring a potential understudied role of MeCP2 dosage in neural progenitors, with implications towards therapies in MeCP2-related disorders. However, the overall message that neurons are tolerant of MeCP2 dosage is overstated given the data presented and the body of literature.

Major comments:

1. While the number of differentially expressed genes and their magnitude are no doubt different between the NPCs and Ns, some additional context is needed to interpret the Ns differential gene expression. Given that cultured neurons are quite immature and tend to have low levels of non-CG methylation, and that non-CG methylation (particularly mCA) has a strong influence on MeCP2-dependent gene regulation (Gabel et al. Nature 2015), what is the non-CG methylation status (and differences) between NPCs and Ns? For example, if the cultured Ns have no mCH (H = ACT), how relevant would the lessons learned from this study bear on mature neurons in vivo. Bisulfite

sequencing would help provide clarity on these questions and help to determine if known trends with mCH and MeCP2 are observed in vitro between NPCs and Ns.

Answer:
We thank the reviewer for raising this important point. Below, we clarify the methylation context of our in vitro system, report additional methylation data we have now included, and discuss how these observations bear on the interpretation of our transcriptional and functional results.
1. Published evidence for mCH accumulation in cultured neurons. A recent study reports that non-CG methylation (particularly mCA) accumulates in primary neuronal cultures with time *in vitro* (Martin et al., 2023). We have added this citation and brief discussion on this important aspect in the revised manuscript.
2. Our additional methylation assay (dot blot). To directly assess methylation in our preparations, we performed dot blot analysis for mCA and included these data in the revised Supplementary Figure 2B. These data show that neurons at the 7-day time point used in our assays display mCA at a lower level than neurons matured for 14 days *in vitro* and the adult mouse cortex (included as a positive control). Thus, consistent with the literature, the 7-day *in vitro* neurons are less methylated in the non-CG context than more mature neurons or adult tissue.
3. Interpretation relative to our transcriptional and functional results. Despite the lower mCA levels at the 7-day time point, two lines of evidence suggest that the main conclusions of our study remain valid and are not solely driven by mCH differences between NPCs and Ns:
- *In vivo* relevance: in our previous *in vivo* study (Luoni et al., 2020), we overexpressed Mecp2 in a WT mouse cortex and did not observe a broad transcriptional deregulation. This outcome is concordant with the minimal transcriptional changes observed in our *in vitro* neurons in the current study.
- Functional readout: as shown in Fig. 7, Mecp2 overexpression did not produce detectable alterations in synaptic function in neurons, further supporting the notion that neuronal overexpression under the tested conditions has limited functional impact despite the lower mCA observed at 7 days.

In summary, we agree that the role of non-CG methylation (mCH/mCA) in MeCP2-dependent regulation is biologically important and intriguing. However, our data indicate that the striking difference in transcriptional response to Mecp2 overexpression is principally associated with cellular state (NPC vs neuron) rather than simply the absolute level of mCA at the examined time point. Nevertheless, we acknowledge that more comprehensive base-resolution methylome data would strengthen our conclusions, but were not practically feasible within the current study.
In the revised manuscript, we have expanded the Discussion to explicitly acknowledge this limitation and to state that future WGBS experiments will be required to fully determine how mCH differences influence MeCP2 binding and downstream transcriptional effects in these models. We hope these additions and clarifications adequately address the reviewer's concern.

2. The MeCP2 CUT&TAG data, measuring both endogenous and exogenous MeCP2 binding, is elegantly designed. However, the localization of MeCP2 to CpG islands is only partially the story. Given the recent exciting evidence that MeCP2 binds in hotspots and non-methylated DNA, it would be important to analyze the data generated in this study to investigate if non-methyl binding or binding in intragenic regions is different between endogenous and exogenous MeCP2 and if that binding pattern is different between NPCs and Ns.

Answer:
We thank the reviewer for this insightful comment. To address this point, we analyzed the CUT&Tag signal for both endogenous and exogenous Mecp2 in NPCs and neurons, dividing the genome into promoter, gene body, and intergenic regions (new data shown in Suppl. Fig. 2). This analysis

confirmed that Mecp2 binding is predominantly enriched at promoter regions, particularly in neurons, where this preference is even more pronounced. In NPCs, the difference was less marked, and Mecp2-exo showed a relatively broader distribution, with Mecp2-exo enrichment also observed within gene bodies and intergenic regions. However, when these data were integrated with our RNA-seq results, only Mecp2 enrichment at promoter regions correlated with gene expression changes, whereas enrichment within gene bodies did not show a significant association with transcriptional regulation (Suppl. Fig. 2D).

3. The notion of subtle gene expression changes from acute MeCP2 increases in neurons has been observed before (Ito Ishida et al. J Neurosci. 2021). In Ito Ishida et al, the authors used focal AAV mediated MECP2 overexpression postnatally and performed behavior and transcriptomics. The authors should compare the transcriptomic signature generated in this study (Figure 12, see "low", "medium", "high" overexpression in DEG number) to the profile generated in neurons in this study. It would be important to dissect the similarities or differences of acute MECP2 dosage on neurons that matured in vitro versus in vivo. Furthermore, despite the subtle molecular changes observed in the "low" MeCP2 overexpression population, Ito-Ishida et al. still observed behavioral deficits overall due to MeCP2 overexpression (Figure 11). Though the electrophysiology in this study is strong, it would be important and helpful to benchmark the behavioral consequence of IUE vs AAV-PHP.eB-MECP2 overexpression. The authors should perform a standard battery of tests to assess to what extent (if at all) IUE MECP2 or AAV-PHP.eB-MECP2 overexpression phenocopies MECP2 duplication mice.

Answer:
In Ito-Ishida et al. (J Neurosci, 2021), MeCP2 was overexpressed in the prefrontal cortex using AAV vectors, leading to highly variable expression levels among transduced neurons. In particular, the authors reported that total Mecp2 reads were approximately 2-, 5-, and 25-fold higher in the GFP-low, GFP-med, and GFP-high populations, respectively, and that the number of DEGs increased dramatically with MeCP2 dosage (25, 302, and 2114 DEGs, respectively; Fig. 12C–D). In our study, Mecp2 was overexpressed approximately two- to threefold, comparable to the *GFP-low* condition in *Ito-Ishida et al.*, and we similarly observed a limited number of transcriptional changes in neurons (44 DEGs with FC < 1, compared to 25 DEGs in 2× Mecp2 neurons in *Ito-Ishida et al.*). These consistent findings reinforce the notion that mild MeCP2 overexpression elicits only subtle transcriptional effects in neurons, both *in vivo* and *in vitro*. The behavioral alterations reported by *Ito-Ishida et al.* likely stem from the presence of cells with much higher MeCP2 levels (up to 25×), which are not representative of the more physiologically relevant and gene therapy–compatible levels achieved in our study.
Regarding behavioral analyses, we have previously shown that a ~2-fold increase in Mecp2 expression using systemic AAV-PHP.eB delivery does not produce detectable behavioral abnormalities in adult mice (Luoni et al., eLife 2023). For the IUE-mediated model, behavioral testing would not be informative, as only a limited subset of cortical neurons is transfected, precluding meaningful behavioral assessment at the organismal level.

4. The results in Figure 6 are intriguing but incomplete. Its not overtly clear why excess MeCP2 should have differential affinity or targeted for degradation given that MeCP2 is not static on DNA (Piccolo et al. eLife 2019). The authors should test if post-translational modifications (e.g., phosphorylation) on the exogenous MeCP2 differentiate the endogenous and exogenous "isoforms". Even if negative, these data would be powerful in determining if exogenous MeCP2 behaves the same biochemically as endogenous MeCP2 in the protein-protein networks (beyond DNA binding as wonderfully measured in this paper already).

Answer:
We fully agree with the reviewer that investigating post-translational differences between endogenous and exogenous Mecp2 would be highly informative and could help elucidate the mechanisms underlying their distinct stability and binding behaviors. However, we believe that a comprehensive biochemical characterization of Mecp2 post-translational modifications deserves a dedicated study.
Nevertheless, to begin addressing this important point, we analyzed by Western blot two of the most characterized Mecp2 phosphorylation sites—Ser80 and Ser421. Interestingly, we observed that phosphorylation at Ser421, which is typically associated with neuronal activity, was reduced in both NPCs and neurons overexpressing Mecp2 (Suppl. Fig. 6). In NPCs, low Ser421 phosphorylation has been linked to reduced proliferation (Li et al., 2014; PMID: 25420914), although such reduction does not appear to affect Mecp2 localization or its chromatin binding capacity (Schmidt et al., 2022; PMID: 36172281).
Conversely, Ser80 phosphorylation displayed an opposite pattern, being markedly reduced in NPCs but increased in neurons upon Mecp2 overexpression. Recent evidence has associated Ser80 phosphorylation with faster chromatin turnover (i.e., weaker DNA binding) (Schmidt et al., 2022; PMID: 36172281), which is consistent with our observation that Mecp2-exo shows lower chromatin affinity in neurons.
Although preliminary, these data are fully consistent with our findings and support the notion that differential Mecp2 phosphorylation could contribute to the distinct biochemical behavior of endogenous and exogenous Mecp2 observed in our models. However, a more comprehensive characterization of Mecp2 post-translational modifications will be essential to fully elucidate the molecular mechanisms underlying these differences.

5. MeCP2 protein levels have a had a lagging effect on gene expression in vivo and in vivo (Bajikar et al. Neuron 2025, Shao et al. Sci Transl Med 2021, Bajikar et al. Human Mol Genet 2025). 5 days between viral transduction and downstream RNA changes is on the same time scale as the "earliest" timepoints where changes were observed in these studies. Additionally, MeCP2 levels rise during neuronal maturation (Shao et al. Genes Dev 2021, Bajikar et al. Human Mol Genet 2025). The authors could expound on the context of their study by assessing MeCP2 levels overtime with their culture protocol and assessing the transcriptome at a later time point (e.g., 4 weeks) after infection. One could hypothesize the N transcriptome will progressively dysregulate matching in vivo expectations, whereas the NPCs could be more stable or adapt (or indeed still remain more susceptible to increased MeCP2 dosage!).

Answer:
We fully agree that assessing the temporal dynamics of Mecp2-dependent transcriptional changes would provide valuable insight. However, extending the experimental timeline to 4 weeks is technically challenging in both cellular models used in this study. Neurons maintained in culture for such prolonged periods typically exhibit declining health and viability, which would confound the interpretation of transcriptomic data. Conversely, NPCs overexpressing Mecp2 display reduced proliferation, as shown in Suppl. Fig.1B, and their cultures could become overrun by non-transduced cells over time, making long-term analyses unreliable.
Nevertheless, in the revised version of the manuscript, we included additional data showing that human NPCs exhibit transcriptional alterations highly similar to those observed in the murine model (Fig. 7D-E). Moreover, *in vivo* experiments (Fig. 7A-B) confirmed that neurons differentiated from Mecp2-overexpressing NPCs display synaptic abnormalities, indicating that the molecular effects persist across systems.
In the same experimental context (Fig. 7A,C), but upon Mecp2 overexpression in the adult mouse brain, we analyzed neuronal physiology two weeks after viral delivery and did not detect any electrophysiological alterations. Furthermore, in our previous work (Luoni et al., 2020), RNA-seq

analysis of cortical tissue from WT mice overexpressing Mecp2 several months after transduction also revealed no major transcriptional changes.
Nevertheless, long-term predictions remain limited, as argued in the Discussion section.

6. The phrase “no effects” in mature neurons is overstated, as transcriptional dysregulation is not the only disrupted output in Mecp2 mutant neurons. There are many phenotypes that were not assessed in vitro or in vivo (e.g., morphology, circuit level disruptions etc). More caution is needed throughout the manuscript, unless these non-molecular phenotypic experiments are performed.

Answer:
We agree that the statement “no effects” in mature neurons was overstated, as additional phenotypes beyond transcriptional regulation (e.g., morphological or circuit-level alterations) were not assessed in our study. In the revised version of the manuscript, we have rephrased this conclusion to indicate that Mecp2 overexpression exerts minor or limited effects in mature neurons, within the assays performed, rather than implying a complete absence of phenotype.

Minor comments:

1. Please provide the differential gene expression statistics/output for all detected genes and denote the direction of fold change to provide as a complete resource for the field.

Answer:
For each RNA-seq dataset included in the study, we have provided comprehensive supplementary tables listing all detected genes and DEGs, including fold-change direction and statistical values. These files constitute the full differential expression output and are available as a resource for the field.

2. Please provide a blot of the NPC and N MeCP2 levels on the same blot for visual/quantitative comparison. This was somewhat gleaned from the CUT&TAG data, but the range of the displays was not always readable.

Answer:
We thank the reviewer for the suggestion. We have now included a representative Western blot comparing total Mecp2 levels in EGFP-control and Mecp2-overexpressing NPCs and neurons on the same membrane (new Suppl. Fig.1A), allowing a direct visual and quantitative comparison between the two cell types.

3. The methods section is incomplete, particularly for the bioinformatics section. Package software versions are left as placeholders.

Answer:
All the missing methodological details have now been corrected and clarified throughout the Materials and Methods section.

4. Please ensure the scale and labels for all genome browser tracks are legible.

Answer:

We have revised all genome browser tracks to ensure that the scales and labels are clearly legible in the updated figures.

5. The axis labels for Figure 6A,B do not agree with the labels on the blot. Please put the correct values as appropriate.

Answer:
We apologize for the oversight; this has been corrected in the revised manuscript.

6. The citations on line 91 for studies that used “supraphysiological viral doses” does not appear to correspond to the appropriate references at the end. These must be critically fixed for readers to find these papers easily.

7. There is a typo on line 372, “MSD” should be MDS

Answer:
We apologize for these oversights. All references and typos have been corrected in the revised manuscript.

Title: MeCP2 gene dosage-dependent neurodevelopmentally restricted defects arise by SWI/SNF-mediated aberrant activation of cell fate-determining bivalent genes

Authors: Mirko Luoni, Michal Kubacki, Serena Gea Giannelli, Francesca Morosi, Claudia Di Berardino, Angelo Iannielli, Alessandro Sessa, Gaia Colasante, Emmanuele Di Patrizio Soldateschi, Chiara Lanzuolo, Vania Broccoli

Reviewer #1 (Remarks to the Author):

I have carefully reviewed the authors' point-by-point responses as well as the updated text and figures. All of my previous concerns have been addressed to my satisfaction. Overall, the revisions have strengthened the manuscript considerably. In particular, the added methodological details and clarifications substantially improve the transparency and reproducibility of the work, and the revised Materials and Methods section is now much clearer and easier to follow. Before final acceptance, I would ask the authors to carefully proofread the manuscript once more for consistency and clarity. Specifically, please verify that the correction of the in utero electroporation timing from E13.5 to E14.5 has been implemented consistently throughout the text, figure legends, and especially in Figure 7.

Answer:

We thank the reviewer for the positive evaluation of the revised manuscript. We have carefully proofread the entire text and corrected the in utero electroporation timing consistently throughout the manuscript, including the figure legends and Figure 7. We appreciate the reviewer's constructive feedback, which has helped further improve the clarity and quality of the work.

Reviewer #2 (Remarks to the Author):

The authors have addressed all the concerns raised by this reviewer. The new Fig 6D shows equivalent half-life for the Mecp2-Exo-v5 tag in both cell types as expected.

James Ellis - Reviewer 2

Answer:

We thank the reviewer for the constructive evaluation and helpful feedback.

Reviewer #3 (Remarks to the Author):

The authors have addressed my concerns by performing additional experiments and analysis to strengthen their conclusions, while also appropriately tempering interpretations where needed. I have a minor suggestion to improve the manuscript: the labels on several graph axes, legends, or scales are too small and not readily legible especially on a printout (e.g., Figure 1c, Figure 2c-e, Figure 3b,c,i, Figure 4f-j, Figure 5e-f, Figure 7d). The authors could consider resizing the labels on these graphs.

Answer:

We thank the reviewer for the constructive evaluation and helpful feedback. We have implemented all the suggested changes and increased the size of the axis labels, legends, and scales in the indicated figures to improve readability, particularly for printed versions of the manuscript.