THE EMBO JOURNAL

De novo DNA methylation controls neuronal maturation during adult hippocampal neurogenesis

Sara Zocher, Rupert Overall, Gabriel Berdugo-Vega, Nicole Rund, Anne Karasinsky, Vijay Adusumilli, Christina Steinhauer, Sina Scheibenstock, Kristian Händler, Joachim Schultze, Federico Calegari, and Gerd Kempermann
DOI: 10.15252/embj.2020107100

Corresponding author(s): Sara Zocher (sarazocher@neuro.mpg.de) , Gerd Kempermann (gerd.kempermann@dzne.de)

Review Timeline:

Submission Date:	20th Oct 20
Editorial Decision:	11th Jan 21
Revision Received:	16th Mar 21
Editorial Decision:	15th Jun 21
Revision Received:	28th Jun 21
Accepted:	2nd Jul 21

Editor: Karin Dumstrei

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision 11th Jan 2021

Dear Sara,

Thank you for submitting your manuscript to the EMBO Journal. I am very sorry for the delay in getting back to you with a decision, but I have now received the complete set of referee reports on this paper.

As you can see from the comments below, the referees find the analysis interesting, but also find that significant revisions are needed to consider publication here. They raise a number of constructive comments and if you should be able to address them then I would like to invite you to submit a revised manuscript. I think it would be helpful to discuss the raised points further and I am happy to do so via phone, email or video. Let me know what works best for you.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

I thank you for the opportunity to consider your work for publication. I look forward to discussing your revisions further

Yours sincerely,

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

Please make sure you upload a letter of response to the referees' comments together with the revised manuscript.

Please also check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
https://bit.ly/EMBOPressFigurePreparationGuideline

IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)

- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/authorguide).
- Expanded View files (replacing Supplementary Information)
Please see out instructions to authors
https://www.embopress.org/page/journal/14602075/authorguide#expandedview

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

Further information is available in our Guide For Authors:
https://www.embopress.org/page/journal/14602075/authorguide

The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 11th Apr 2021.

https://emboj.msubmit.net/cgi-bin/main.plex

Referee #1:

Zocher et al.: "De novo DNA methylation controls neuronal maturation during adult hippocampal neurogenesis"

Dynamic changes in DNA methylation have been found to significantly contribute to (neuro-) developmental processes in particular to cell-type specification and differentiation. This manuscript uses state-of-the-art genomic and transcriptomic analyses combined with histological and behavioral analyses to investigate how de novo methylation catalyzed by DNMT3a and/or DNMT3b regulates the generation of new neurons in the hippocampus of adult mice. Reduced Representation Bisulfite Sequencing of FACS-isolated cells of the hippocampal neurogenic lineage (Stem / progenitor cells; late progenitor cells; neurons) revealed dynamic changes in methylation pattern in particular between late progenitor cells and neurons. Interestingly these changes are enriched on genes related to neuronal maturation and synaptic function. A very surprising observation of this paper is the finding that conditional removal of DNMT3a and DNMT3b does not result in significant perturbation of the rate of hippocampal neurogenesis - neither under standard housing conditions nor in the context of enriched environment, which is a well described positive behavioral stimulus for the rate of neurogenesis and which has been found to modify methylation patterns in the hippocampus. Marker expression analysis (DCX and Calbindin) and morphological analysis of dendrites and spines suggest that loss of DNMT3a/b impairs maturation. The authors also analyze the expression of immediate early genes in the dentate gyrus and the CA3 region following short term exposure to an enriched environment and perform behavioral analyses, and reveal less cfos positive neurons in the DG and CA3 region in knockout animals and impaired cognitive flexibility in the Morris-Water-Maze.
Over all this is a well-conceived study that a) potentially (the authors have not submitted the sequencing data for evaluation by the reviewers) provides an important resource on developmental

stage-specific DNA methylation in adult hippocampal neurogenesis, b) describes for the first time the function of DNMT3a/b in adult hippocampal neurogenesis, c) uncovers an uncoupling of neuronal cell-fate decisions from de novo methylation.

I do have a few major comments:
1. The authors perform in vitro experiments to analyze the impact of DNMT3a/b knockout on adult neural stem/progenitor differentiation and neuronal maturation. While it may be consistent with the later in vivo observation to focus on neuronal morphology, I would like to suggest that the authors also quantify the neuronal and astrocytic differentiation rate, in particular because the representative images in Figure 2D suggest differences in the percentage astrocyte generation. Astrocyte morphologies also seem to be affected by the KO, and it would be interesting to further document this phenomenon or at least comment on it.
2. I assume that the authors are documenting the section "Deletion of de novo DNA methyltransferases in neural precursor cells impairs maturation of new-born neurons in vivo" in supplemental figure 5. Please correct the typo. On page 10 the authors write that "... no differences in total numbers of neural precursor cells and their neuronal progeny were found between KO and WT mice three months after tamoxifen administration (Fig. S6D-G)...." It is my understanding that Figure S5G provides a quantification of BrdU cells 3 months after the pulse-label and recombination. To substantiate the statement that the total number of neuronal progeny was unaffected the authors should phenotype the BrdU+ cells. In the context of the impaired maturation it would also be important to use the 3 months time-point to evaluate whether newborn cells ultimately downregulate immature markers and upregulate mature markers in the DNMT3a/b mutants.
3. The authors analyze immediate early gene expression following short-term enriched environment. While the phenomenon that immediate early gene expression is reduced is interesting, it raises a number of additional questions: The DNMT3a/b knockout is induced specifically in adult-born neurons. Are adult-born DNMT3-deficient neurons less likely to be activated or is this a network effect? Birthdating adult born neurons with BrdU and analyzing the expression in this cell population would provide some more insight. It is also surprising - given the findings in particular of the Hen laboratory that adult-born neurons may promote sparse activation of the DG by a negative feedback loop - that the potentially less mature adult-born neurons in the DNMT3a/b KO are associated with lesser activity. Please discuss.
4. For evaluation of the manuscript it is important to provide the sequencing data.

Minor comments:
5. The authors use the term pro-neuronal transcription factors somewhat deliberately. I would like to suggest that the authors use a different term as it is reminiscent of the term pro-neural transcription factors, which is mainly used for neurogenic bHLH transcription factors. I am not aware that e.g. egr4 and Npas2 have a pro-neuronal function in the sense of promoting neuronal differentiation.
6. The authors may want to consider to better highlight in the abstract the interesting finding that de novo methylation catalyzed by DNMT3a/b does not affect fate choice of adult neural stem cells.

Referee #2:

In this paper, Zocher el al. combined multiple technical approaches (Reduced Representation Bisulfite Sequencing, single cell RNA sequencing, genetically modified animals, in vitro and in vivo experiments, immunohistology, and animal behavior) to study the role of de novo DNA methylation

in adult hippocampal neurogenesis. The authors first report that de novo DNA methylation of neuronal genes underlies adult hippocampal neurogenesis. Then, they demonstrate that the deletion of the de novo methyltransferases Dnmt3a and Dnmt3b impairs neuronal maturation in vitro and prevents neurogenesis-associated do novo DNA methylation. Next, they show that the in vivo conditional and inducible deletion of Dnmt3a and Dnmt3b in neuronal stem cells only does not affect the formation of new neurons but impairs their maturation, and that neurogenesis-associated de novo DNA methylated genes tend to be upregulated during neuronal formation. Finally, the authors report that the deletion of Dnmt3a and Dnmt3b in adult NSCs reduces experience-dependent activation of the hippocampus, and impairs cognitive flexibility in a hippocampus-dependent memory task. One of the most important contributions of the manuscript are the conditional DNMT KO mouse models and its histological and behavioral evaluation. These aspects are very well conducted and convincing. Zocher et al. show that the induction of neurogenesis is not dependent on methylation, while integration and maturation of neurons is.

Given the importance of adult neurogenesis in the normal and disease brain states, a better understanding of the molecular mechanisms controlling the formation, maturation, and integration of adult born dentate granule cells is critical. From this perspective, this study from Zocher et al. reporting that only the maturation of adult born new neurons (and not their generation) depends on de novo DNA methylation reports new and interesting findings and improves our understanding of the epigenetic control of adult hippocampal neurogenesis. Overall, the study is well designed, presents a nice combination of methods to answer the raised question and the amount of work is significant. However, some major and minor issues remain and must be improved before publication, including improvements to their molecular studies, especially the DNA methylation and single cell expression analyses, as the methodologies used and the amount of data presented is insufficient to address the questions appropriately.

Major concerns:

1. To identify the changes in DNA methylation during adult hippocampal neurogenesis, Zocher et al. rely on Reduced Representation Bisulfite Sequencing. While this methodology allows the profiling of DNA methylation levels of thousands of CpG sites in parallel and at the single-nucleotide resolution, it samples only 10-15% of all the CpG sites of the genome. As only a fraction of the genome is covered, global methylation statistics can not be called from RRBS data. Furthermore, CpH methylation is under represented, making any description of it very challenging. Consequently, there is a risk that the results reported here might be due to this limited coverage of genomic CpGs/genes, which underpowers the final conclusions. If the full set of experiments cannot be reprocessed using whole genome bisulfite sequencing (WGBS) experiments, at least the first series of experiments identifying the neurogenesis-related changes in DNA methylation should be repeated for WGBS and compared to RRBS data to demonstrate that RRBS data can indeed provide reliable data related to changes during adult neurogenesis. In addition, this limitation should be clearly stated in the result section, and a detailed paragraph on this limitation should be included in the discussion section.

2. Covered genes: please elaborate how you defined which genes were covered by the RRBS data: is it required that a certain portion of the gene body is covered, or does it just require that any reads fall within a gene? Please give details of where methylation changes were observed: enhancers, promoters, introns etc.

3. Gene enrichment analyses: which method was used for the initial analysis (Fig 1E)? SYNGO showed enrichment of 148 genes compared to 102 genes by random chance, which is not many above chance. No details were given regarding how many of the 1,670 MANGO entries were found in de novo methylated genes. What are the results for comparing all genes covered by RRBS to these gene lists, is there any enrichment for the subset of covered genes? The conclusion taken from the use of SYNGO (page 7 l. 1 and page 9, l. 16) should be modified. Indeed, all the GO terms in this dataset are related to synaptic functions (as all the genes in this data set have been curated for their role in synaptic mechanisms) so the "enrichment in sub-ontologies related to synaptogenesis and synaptic function" is obvious. A full list of the GO enriched GO terms should be provided as a supplementary figure/table.

4. From Fig 2 D, differentiation of wildtype and DNMT-KO NPCs, it seems that the deletion of de novo methyltransferases biases the progeny of neurospheres towards astrocytes. A quantification of cell types is required, including a more detailed characterization of the ratio of glia vs neurons, and a comparison with the data obtained in vivo (Fig S5 B-C) should be provided. Also, based on the location of DAPI signals, a lot of cells in the WT sample are negative for Tbb3 and GFAP, while all cells in the KO are stained. Astrocytes show different morphologies between samples, with large spread-out cells in the KO, and thinner smaller cells in the WT. The two shown images are very different in many aspects, which makes comparison between the WT and KO difficult. In addition, quantification of dendritic lengths should be easy to perform and would be a valuable addition to the data.

5. The histology experiments are well designed and the reported data are coherent. However, the information regarding the methodology for these experiments is insufficient. For each set of experiments, the authors must provide the number of sections that were counted, the number of animals used, the regions (dorsal and/or ventral hippocampus? Dorsal and/or ventral blade of the DG?) and representative photomicrographs of the different stainings must be provided (for example for Fig 4A-B which are lacking), in Supplementary Data if needed. For the study of the dendritic spines: from how many cells taken from how many animals were these data generated? The dendrites from the molecular layer were analyzed, but did the authors keep constant the dendritic order they analyzed (as spine density can vary depending on dendritic order)?

6. Neuronal morphology of WT and KO neurons: while there are dramatic differences in morphology between cell culture generated neurons (Fig 2F), the same analysis in vivo showed far less differences. This might hint at non-cell autonomous effects that the surrounding tissue might have on newly generated neurons. Please discuss.

7. The number of cells for single cell expression analysis is low which affects the experimental power. The authors admitted to not having found any mature neurons in their samples which is very likely due to loss of elongated neurons during cell isolation from the tissue. Instead of isolating whole cells for single cell RNA-seq, the authors could attempt to prepare nuclei from brains and perform single nuclei RNA-seq. If it is not possible to get GFP-positive nuclei due to cytoplasmic location of the reporter, it may be possible to sort for BrdU, followed by NeuN and other transcription factors. Preferably the analysis would be extended to analyze several thousand nuclei as is typical for many single cell/nucleus studies now, using more brains if necessary. This would provide more insights into deficits in maturation in the KO, improve the correlation of methylation and gene expression, and allow the calling of differentially expressed genes between WT and KO.

8. The analysis/representation of single-cell data needs to be improved. First, basic QC metrics for single cell analysis must be given (mean number of reads/genes per cell, number of genes detected,

% of sequencing saturation, fraction of reads mapping to mitochondrial gene and transcriptome etc, and a plot showing which cells were derived from which sample). Furthermore, from how many animals were these single cells obtained? The authors should also represent the t-SNE colored by individuals and genotype. A legend for the cluster identity should be included in Fig 5A. Alternatively, and because the number of clusters is limited, the authors could replace "T1-4b" by cluster names (in Fig 5A-D). The genes in Fig 5B should be reorganized vertically to match with developmental dynamics (from NSCs to immature neurons from top to bottom). The log fold change in Fig 5D and 5E must be specified.

9. How many genes in total are detected in both the single cell expression analysis and the DNA methylation data? About 100 genes are shown in Fig 5D, but it is not clear if these are only the de novo methylated genes with expression changes or all the de novo methylated genes.

10. The correlation of transcriptomic data from a limited number of cells with DNA methylation data from a method only covering a fraction of the genes is difficult and does not allow one to draw general conclusions about the effects of de novo methylation on neuronal gene expression. A method for whole genome coverage of DNA methylation should be used, e.g. WGBS to include more genes for the analysis and to allow investigation of CpH methylation changes.

11. Very little analysis of the ENR-induced DNA methylation changes in the different populations was undertaken. For example, what genomic features are they located in? What genes are they associated with? Are they associated with regulatory elements, and if so, are there particular associated enriched TF motifs?

12. The authors show that KO mice showed significantly fewer c-Fos-positive cells in the dentate gyrus and CA3 of the hippocampus after stimulation in ENR (Fig. 6D-F). It would be interesting to know if this decrease of c-FOS staining is due to a cellular autonomous mechanism (only newly generated neurons are less activated during ENR), or if the KO of Dnmts in NSCs has a more systemic effect on DG activity. To assess this, the authors should look at the double staining of BrdU + cFos to understand whether the observed decrease of c-FOS staining is only due to a lower activation of new born neurons or a more general reduced activity of the DG.

Minor concerns:

- Are the percentages of CpGs that change methylation state listed on pages 5 and 9 the fraction of all sufficiently covered CpGs (based on threshold criteria outlined in methods), or all CpGs in the genome? It would be helpful to clarify this in the main text.

- Fig 1C : the legend on the Y axis is missing.

- Fig 1F: a legend for the size of the dots should be added (I guess it represents the number of genes per GO term).

- Fig S1H shows the dynamics of markers across neurogenesis stages (NSCs, IPCs, Neurons). The represented markers are all NSCs markers that are downregulated during neurogenesis. It would be good to include other markers that are upregulated during neurogenesis (such as Calb2, Rbfox3...for which the data is only presented regarding DCX expression - Fig S1G).

- Fig S1G: why do the different genes have a different number of replicates? Also, given the low number of replicates (2 to 3), the use of a parametric test (such as ANOVA) should be avoided and non-parametric tests should be used.

- How were the methylation levels shown in Fig S2 C and D calculated? Was it all methylated CpG (or CpH) basecalls divided by total basecalls at CpG (or CpH) positions? Rather than plotting the median methylation level, which is challenging to interpret, it would be more useful to plot the distributions.

- Typo in Fig 3E label.

- How have the neuronal enhancers and super-enhancers used here been defined? Please provide a table as supplementary data. Which are these super enhancers and why are they neuronal related? Some more explanation is needed.

- Fig 4C is confusing, and could be removed or converted to a landscape orientation. Also time points should be in weeks.

- Does Fig 4E show WT or KO mouse?

- Fig 4E: small squares should be added to the overlaid picture to identify cells that were magnified on the bottom panel.

- Fig 4G: the p-value from the 2-way ANOVA indicates a significant effect. However, the different p-values for the 2 main effects (Distance to soma and Genotype) must be reported. Indeed, it seems that the reported p-value is driven by the effect of the Distance to soma and not by the Genotype. Did the authors forget to include the results of the post-hoc tests?

- Fig 6 C: Representative photomicrographs of the STD conditions (WT and KO) must be included.

- Fig 6 E-F: Given the low number of replicates (5 max per condition), parametric tests such as t-test cannot be used. A non-parametric test must be used instead.

- Fig 7: how many animals were used per group? Same comment as above, parametric tests are probably not usable (as normality cannot be tested for less than 20-30 samples per condition).

- Fig S5: Could a panel similar to D be added for experiments presented in B-C? The GFP panel for the astrocytes in panel C must be included, and small squares in the main overlay must be added to highlight magnified cells. In the legend, "D" and "G" letters must be put in bold.

- Fig S8F: The discrimination index indicates that neither the WT nor the KO animals spent more time exploring the new object compared to the old one. This is not what is expected for an object recognition test and could indicate a lack of memory for both genotypes. Please check and comment whether this lack of preference for the novel object is observed for both groups.

- Page 5, l. 14: the reference for Boyle et al. should be replaced by the appropriate number. Similar changes have to be made on page 19, l. 2 and 22).

- Page 6, l. 3: the full list of enriched motifs for de novo methylated CpGs should be presented in a supplementary table.

- Page 7, l. 7: the word "synaptic plasticity" should be rephrased as there is nothing in the data presented that links these genes to synaptic plasticity (which is a specific and well documented physiological phenomenon).

- Page 10, l. 15 and l. 17: Shouldn't it be Fig. S5 and not Fig. S6?

- Page 20: for the drugs used, please add the catalogue number and the concentration to which they were dissolved (prior to injection).

- Page 22, last line: the last word of the line ("and") must be deleted.

- Page 24, l. 4: were the brains transcardially perfused with sodium chloride only? In general, for immunohistochemistry, animals are perfused with PFA following saline perfusion in order to directly, immediately and completely fix brain tissue. Please check and clarify.

- Page 25, please include to the table the references and dilutions of the secondary antibodies.

- Page 27, l. 18: The size of the open field is probably not 50 cm2 but 50cm*50cm.

Referee #3:

The manuscript interrogates the role of de novo DNA methyltransferases Dnmt3a and Dnmt3b in adult neurogenesis and brain function using elegant mouse genetics, DNA methylome mapping and behavioral studies. In general the work is done with rigor and presented with clarity.
While the manuscript provides a careful dissection of the role of Dnmt3a/b in neurogenesis and hippocampal function, the major weakness is that it does not provide molecular insights by identifying genes regulated by DNA methylation in neurogenesis. Therefore the manuscript brings little conceptual advances compared to previous work that already demonstrated the importance of Dnmt3 enzymes for mouse neurogenesis.

Major points:

-The overall originality of the study is uncertain given that several previous studies combined Dnmt3a-2lox mice with Nestin-Cre or Nestin-CreERT2 for conditional deletion in neural progenitor cells. The authors should better explain how their study complements previous work and addresses novel unsolved questions.

-More information is needed about the quality of the RRBS datasets: sequencing depth, number of covered CpGs, number of replicates, correlation between replicates, etc...Without this information it is impossible to evaluate the quality of the datasets on which are based the manuscript's conclusions.

-I am a bit skeptical about the motif analysis in Figure 1D because it looks like it simply reflects the enrichment of the CG dinucleotide in sites of methylation.

-It is important to know whether there are global changes in CpH and CpG methylation during

neurogenesis. In contrast to what is claimed in the text, Figure S2D does not seem to address this because it shows methylation of an unknown set of 765 CpGs. Average CpG methylation scores of all CpGs should be shown.

-In Figure 3, RRBS should also be performed in in vitro NSPCs before neuronal differentiation. This would allow addressing two important questions:1) to what extent do changes in methylation during in vitro neuronal differentiation recapitulate the in vivo patterns seen in Figure 1? 2) Do hypomethylated CpGs in Dnmt3a/b KO neurons reflect failure in de novo or maintenance methylation?

-The central question of the work is whether de novo methylation events are required to regulate gene transcription events during neurogenesis. Quite disappointingly, the authors found little transcriptional changes in conditional KO animals by single cell RNA-seq and are thus unable to relate aberrant DNA methylation to perturbed gene expression in neurogenesis. However I don't find that this is addressed in a satisfactory manner because the authors do not perform methylome and transcriptome analyses in the same cellular samples. Why not doing RNA-seq on in vitro differentiated neurons to directly relate methylation and gene expression in the same cells?

-Although RRBS provides accurate and quantitative methylation calls, it provides very poor coverage of gene bodies and enhancers, thus limiting data interpretation. WGBS would certainly be much more informative. I acknowledge that WGBS is costly and requires more DNA but it could be done on WT and KO in vitro neurons.

-Explain what is shown on the y axis in Figure 1C.

-Please explain with more details what annotations were used to define enhancers in the study.

-The sequencing data should be made accessible in GEO at time of submission for review purposes.

Author response to Reviewer comments

We thank the reviewers for their constructive feedback. We have addressed the comments by performing additional experiments, futher analyses and by re-writing the manuscript. The detailed changes are outlined below in the point-by-point response.

Referee #1:

Zocher et al.: "De novo DNA methylation controls neuronal maturation during adult hippocampal neurogenesis"

Dynamic changes in DNA methylation have been found to significantly contribute to (neuro-) developmental processes in particular to cell-type specification and differentiation. This manuscript uses state-of-the-art genomic and transcriptomic analyses combined with histological and behavioral analyses to investigate how de novo methylation catalyzed by DNMT3a and/or DNMT3b regulates the generation of new neurons in the hippocampus of adult mice. Reduced Representation Bisulfite Sequencing of FACS-isolated cells of the hippocampal neurogenic lineage (Stem / progenitor cells; late progenitor cells; neurons) revealed dynamic changes in methylation pattern in particular between late progenitor cells and neurons. Interestingly these changes are enriched on genes related to neuronal maturation and synaptic function. A very surprising observation of this paper is the finding that conditional removal of DNMT3a and DNMT3b does not result in significant perturbation of the rate of hippocampal neurogenesis - neither under standard housing conditions nor in the context of enriched environment, which is a well described positive behavioral stimulus for the rate of neurogenesis and which has been found to modify methylation patterns in the hippocampus. Marker expression analysis (DCX and Calbindin) and morphological analysis of dendrites and spines suggest that loss of DNMT3a/b impairs maturation. The authors also analyze the expression of immediate early genes in the dentate gyrus and the CA3 region following short term exposure to an enriched environment and perform behavioral analyses, and reveal less cfos positive neurons in the DG and CA3 region in knockout animals and impaired cognitive flexibility in the Morris-Water-Maze. Over all this is a well-conceived study that a) potentially (the authors have not submitted the sequencing data for evaluation by the reviewers) provides an important resource on developmental stage-specific DNA methylation in adult hippocampal neurogenesis, b) describes for the first time the function of DNMT3a/b in adult hippocampal neurogenesis, c) uncovers an uncoupling of neuronal cell-fate decisions from de novo methylation.

I do have a few major comments:
1. The authors perform in vitro experiments to analyze the impact of DNMT3a/b knockout on adult neural stem/progenitor differentiation and neuronal maturation. While it may be consistent with the later in vivo observation to focus on neuronal morphology, I would like to suggest that the authors also quantify the neuronal and astrocytic differentiation rate, in particular because the representative images in Figure 2D suggest differences in the percentage astrocyte generation. Astrocyte morphologies also seem to be affected by the KO, and it would be interesting to further document this phenomenon or at least comment on it.

We have performed additional analyses on the *in vitro* differentiated cells and present these new results in Fig. 2E-K and Appendix Fig. S2C-F. A description of the data in the main text can be found

on page 8, lines 176-193. We also included a discussion of the comparison between the results obtained from *in vitro* and *in vivo* experiments on page 20, lines 470-492.

2. I assume that the authors are documenting the section "Deletion of de novo DNA methyltransferases in neural precursor cells impairs maturation of new-born neurons in vivo" in supplemental figure 5. Please correct the typo.

We corrected the typo. Thank you for pointing it out. This figure corresponds now to Figure EV3.

On page 10 the authors write that "... no differences in total numbers of neural precursor cells and their neuronal progeny were found between KO and WT mice three months after tamoxifen administration (Fig. S6D-G)...." It is my understanding that Figure S5G provides a quantification of BrdU cells 3 months after the pulse-label and recombination. To substantiate the statement that the total number of neuronal progeny was unaffected the authors should phenotype the BrdU+ cells. In the context of the impaired maturation it would also be important to use the 3 months time-point to evaluate whether newborn cells ultimately downregulate immature markers and upregulate mature markers in the DNMT3a/b mutants.

We phenotyped the BrdU-positive cells for expression of the mature neuronal marker Calbindin and the immature marker Doublecortin. We found that three months after BrdU pulse-label and recombination, the vast majority of BrdU cells (> 85 %) in both genotypes expressed Calbindin but not Doublecortin, identifying them as mature neurons. However, *Dnmt3a/b*-KO mice maintained slightly reduced percentages of mature neurons compared to WT mice. These new results are presented in Figure 5E-F and described in the main text on page 14, lines 312-319.

3. The authors analyze immediate early gene expression following short-term enriched environment. While the phenomenon that immediate early gene expression is reduced is interesting, it raises a number of additional questions: The DNMT3a/b knockout is induced specifically in adult-born neurons. Are adult-born DNMT3-deficient neurons less likely to be activated or is this a network effect? Birthdating adult born neurons with BrdU and analyzing the expression in this cell population would provide some more insight. It is also surprising - given the findings in particular of the Hen laboratory that adult-born neurons may promote sparse activation of the DG by a negative feedback loop - that the potentially less mature adult-born neurons in the DNMT3a/b KO are associated with lesser activity. Please discuss.

To address this question, we have analyzed c-Fos expression in adult-born neurons and further characterized hippocampal activation patterns in *Dnmt3a/b*-KO mice and WT mice. Our new results suggest that the difference in numbers of c-Fos-positive cells in the dentate gyrus of *Dnmt3a/b*-KO mice is not a results of the reduced activation of adult-born neurons themselves, but rather due to increased inhibition on the hippocampal circuitry, potentially due to higher percentages of immature neurons in KO mice. We have included these new findings in Fig. 6F-I and describe them in the main text on page 15, lines 349-376. We discuss additional mechanisms how *Dnmt3a/b*-KO neurons could affect hippocampal activation patters on page 22, lines 505-508.

4. For evaluation of the manuscript it is important to provide the sequencing data.
All sequencing data generated in the study are now deposited at GEO (accession number GSE167955).

Minor comments:
5. The authors use the term pro-neuronal transcription factors somewhat deliberately. I would like to suggest that the authors use a different term as it is reminiscent of the term pro-neural transcription factors, which is mainly used for neurogenic bHLH transcription factors. I am not aware that e.g. egr4 and Npas2 have a pro-neuronal function in the sense of promoting neuronal differentiation.

We had initially chosen the term „pro-neuronal" transcription factor to distinguish it from „pro-neurogenic" transcription factor, but we understand the potential confusion. We now changed it to „neuronal" transcription factor and only refer to transcription factors that are expressed in neurons.

6. The authors may want to consider to better highlight in the abstract the interesting finding that de novo methylation catalyzed by DNMT3a/b does not affect fate choice of adult neural stem cells.

We modified the abstract to better highlight our novel findings. Thanks for the suggestion.

Referee #2:

In this paper, Zocher el al. combined multiple technical approaches (Reduced Representation Bisulfite Sequencing, single cell RNA sequencing, genetically modified animals, in vitro and in vivo experiments, immunohistology, and animal behavior) to study the role of de novo DNA methylation in adult hippocampal neurogenesis. The authors first report that de novo DNA methylation of neuronal genes underlies adult hippocampal neurogenesis. Then, they demonstrate that the deletion of the de novo methyltransferases Dnmt3a and Dnmt3b impairs neuronal maturation in vitro and prevents neurogenesis-associated do novo DNA methylation. Next, they show that the in vivo conditional and inducible deletion of Dnmt3a and Dnmt3b in neuronal stem cells only does not affect the formation of new neurons but impairs their maturation, and that neurogenesis-associated de novo DNA methylated genes tend to be upregulated during neuronal formation. Finally, the authors report that the deletion of Dnmt3a and Dnmt3b in adult NSCs reduces experience-dependent activation of the hippocampus, and impairs cognitive flexibility in a hippocampus-dependent memory task. One of the most important contributions of the manuscript are the conditional DNMT KO mouse models and its histological and behavioral evaluation. These aspects are very well conducted and convincing. Zocher et al. show that the induction of neurogenesis is not dependent on methylation, while integration and maturation of neurons is.

Given the importance of adult neurogenesis in the normal and disease brain states, a better understanding of the molecular mechanisms controlling the formation, maturation, and integration of adult born dentate granule cells is critical. From this perspective, this study from Zocher et al. reporting that only the maturation of adult born new neurons (and not their generation) depends on de novo DNA methylation reports new and interesting findings and improves our understanding of the epigenetic control of adult hippocampal neurogenesis. Overall, the study is well designed, presents a nice combination of methods to answer the raised question and the amount of work is significant. However, some major and minor issues remain and must be improved before publication,

including improvements to their molecular studies, especially the DNA methylation and single cell expression analyses, as the methodologies used and the amount of data presented is insufficient to address the questions appropriately.

Major concerns:

1. To identify the changes in DNA methylation during adult hippocampal neurogenesis, Zocher et al. rely on Reduced Representation Bisulfite Sequencing. While this methodology allows the profiling of DNA methylation levels of thousands of CpG sites in parallel and at the single-nucleotide resolution, it samples only 10-15% of all the CpG sites of the genome. As only a fraction of the genome is covered, global methylation statistics can not be called from RRBS data. Furthermore, CpH methylation is under represented, making any description of it very challenging. Consequently, there is a risk that the results reported here might be due to this limited coverage of genomic CpGs/genes, which underpowers the final conclusions. If the full set of experiments cannot be reprocessed using whole genome bisulfite sequencing (WGBS) experiments, at least the first series of experiments identifying the neurogenesis-related changes in DNA methylation should be repeated for WGBS and compared to RRBS data to demonstrate that RRBS data can indeed provide reliable data related to changes during adult neurogenesis. In addition, this limitation should be clearly stated in the result section, and a detailed paragraph on this limitation should be included in the discussion section.

We agree with the reviewer that it would be ideal to perform WGBS on the different cell populations. However, we want to highlight that preparing bisulfite sequencing libraries from the few NSPCs of the adult hippocampus is very challenging (we obtained 1,500 NSPCs and 3,000 lPCs per mouse) and repeating this experiment with WGBS would, due to the low numbers of cells, likely not yield full genome coverage either. Our study, albeit it does not provide whole genome coverage, contains the first published dataset of DNA methylation profiles of adult NSPCs *in vivo*. We did identify genes that change DNA methylation during adult neurogenesis, but do not claim to provide an exhaustive list of genome-wide DNA methylation changes.

We re-wrote the manuscript to clarify that the main goal of this study was to provide a functional characterization of the role of *de novo* DNA methyltransferases during adult hippocampal neurogenesis (at the cellular level) and not to provide a resource of genome-wide DNA methylation changes and their relationship with gene expression. We successfully used RRBS to validate that there is *de novo* DNA methylation during adult neurogenesis (Figure 1) as well as to confirm that *Dnmt3a/b*-KO leads to hypomethylation in neurons (Figure 3).

To address the reviewer‘s concern, we now clearly state in the results and discussion section the limitations of RRBS compared to WGBS. We mention the reduced coverage of CpHs and that it is not possible to provide global DNA methylation estimates (which we have removed; main text page 6, lines 126-131). We also report the exact numbers of CpGs and CpHs covered by RRBS. Since all of our analyses were performed by calculating enrichment of differentially methylated cytosines/genes over the background list of cytosines/genes covered by RRBS, there should not be a bias of RRBS on the functional enrichment analyses. We discuss that the reported percentages of differentially methylated CpGs could be biased due to the reduced coverage of enhancers by RRBS (page 18, lines 424-442).

2. Covered genes: please elaborate how you defined which genes were covered by the RRBS data: is it required that a certain portion of the gene body is covered, or does it just require that any reads fall within a gene?

We considered genes as „covered by RRBS“ if at least one CpG was covered with at least ten reads in at least three samples per group. We now specified this in the methods section (page 33, line 771-773).

Please give details of where methylation changes were observed: enhancers, promoters, introns etc.

In addition to the relative enrichment over background, we now also included graphs showing the percentages of CpGs located in the different genomic areas in Fig. 1C and Fig. EV1.

3. Gene enrichment analyses: which method was used for the initial analysis (Fig 1E)?

The initial enrichment analysis was performed with pathways annotated in the Reactome knowledge-base (Fabregat *et al*, 2018) using the R package ReactomePA (Yu & He, 2016). We now specificied this in the Figure legends (Fig. 1E, Fig. 3F) and provided further details in the methods section (page 32, lines 765-766).

SYNGO showed enrichment of 148 genes compared to 102 genes by random chance, which is not many above chance.

We performed hypergeometric testing of the 148 *de novo* methylated genes annotated in SYNGO compared with the background list of 768 genes (covered by RRBS and annotated in SYNGO) and found a significant enrichment of genes with SYNGO annotation among the *de novo* methylated genes (p = 0.0028). We included this statistical analysis in the main text on page 7, line 140.

No details were given regarding how many of the 1,670 MANGO entries were found in de novo methylated genes.

Of the 397 genes annotated in MANGO, we selected the ones for which a functional relevance in adult neurogenesis had been demonstrated, which yielded 359 genes of which 203 genes were covered by RRBS. As indicated in Figure 1G, we detected for 46 of those genes *de novo* DNA methylation during neurogenesis. We now mention this number in the main text on page 7, lines 145-146 and provide further description of the MANGO data in the methods section on page 32, lines 767-769.

What are the results for comparing all genes covered by RRBS to these gene lists, is there any enrichment for the subset of covered genes?

All described gene set enrichment analyses were performed with the differentially methylated genes as query list and the list of all genes covered by RRBS as background list. Even if the background list would be enriched in any of the gene sets compared to the whole genome, this would not invalidate our results.

The conclusion taken from the use of SYNGO (page 7 l. 1 and page 9, l. 16) should be modified. Indeed, all the GO terms in this dataset are related to synaptic functions (as all the genes in this data set have been curated for their role in synaptic mechanisms) so the "enrichment in sub-ontologies related to synaptogenesis and synaptic function" is obvious.

We modified the text to avoid misunderstanding. The point was that *de novo* methylated genes were significantly enriched in several terms of both SYNGO sub-ontologies „cellular component/ synaptogenesis“ and „biological process/ synaptic function“ relative to the background list of all RRBS covered genes. The "enrichment in sub-ontologies related to synaptogenesis and synaptic function" is not obvious, but was indicated by a significant enrichment in the respective SYNGO terms.

A full list of the GO enriched GO terms should be provided as a supplementary figure/table.

We now provide a full list of the results from Reactome pathway analyses, SYNGO enrichment and MANGO enrichment in Table EV2.

4. From Fig 2 D, differentiation of wildtype and DNMT-KO NPCs, it seems that the deletion of de novo methyltransferases biases the progeny of neurospheres towards astrocytes. A quantification of cell types is required, including a more detailed characterization of the ratio of glia vs neurons, and a comparison with the data obtained in vivo (Fig S5 B-C) should be provided. Also, based on the location of DAPI signals, a lot of cells in the WT sample are negative for Tbb3 and GFAP, while all cells in the KO are stained. Astrocytes show different morphologies between samples, with large spread-out cells in the KO, and thinner smaller cells in the WT. The two shown images are very different in many aspects, which makes comparison between the WT and KO difficult. In addition, quantification of dendritic lengths should be easy to perform and would be a valuable addition to the data.

We have now quantified total numbers of *in vitro* differentiated neurons, astrocytes and DAPI-positive nuclei and calculated the rate of neuronal and astrocytic differentiation. We further measured total dendritic lengths of differentiated neurons and analyzed the area covered by differentiated astrocytes as a measure of astrocyte morphology. These new results are presented in Fig. 2E-K and Appendix Fig. S3C-F and described in the main text on page 8, lines 176-193. We also included a comparison of *in vitro* and *in vivo* results in the discussion on page 20, lines 470-492.

As suggested by the reviewer, we also determined the percentages of cells that did not express Tubb3 nor Gfap. We detected an increased percentage of unstained cells in WT cultures (Figure R1). However, since the nature of these cells is unclear at this stage, we did not include this Figure in the manuscript.

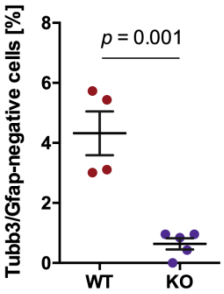

Figure R1: *Dnmt3a/b*-deficient hippocampal precursor cell cultures generated a reduced percentage of Tubb3/GFAP-negative cells during differentiation. Depicted is the percentage of unstained cells among all DAPI-positive nuclei.

5. The histology experiments are well designed and the reported data are coherent. However, the information regarding the methodology for these experiments is insufficient. For each set of experiments, the authors must provide the number of sections that were counted, the number of animals used, the regions (dorsal and/or ventral hippocampus? Dorsal and/or ventral blade of the DG?) and representative photomicrographs of the different stainings must be provided (for example for Fig 4A-B which are lacking), in Supplementary Data if needed.

The revised manuscript includes a separate methods section containing a description of the methodology applied for cell quantifications (page 29, lines 684-692). We further reported detailed stasticial information for every experiment in Appendix Table S5. Representative images for all analyzed stainings were added in the respective Figures.

For the study of the dendritic spines: from how many cells taken from how many animals were these data generated?

Spines were quantified on at least 10 neurons per animal (n = 3 mice, WT; n = 4 mice, KO). Exact numbers have been added to the Figure legend and to Appendix Table S5.

The dendrites from the molecular layer were analyzed, but did the authors keep constant the dendritic order they analyzed (as spine density can vary depending on dendritic order)?

The dendritic order was not considered during spine quantifications. Spines were quantified on randomly chosen dendrites in the molecular layer with the experimenter blinded to the sample genotypes. We added this information to the methods section on page 30, line 700.

6. Neuronal morphology of WT and KO neurons: while there are dramatic differences in morphology between cell culture generated neurons (Fig 2F), the same analysis in vivo showed far less differences. This might hint at non-cell autonomous effects that the surrounding tissue might have on newly generated neurons. Please discuss.

The extracellular signals (cell contacts, ECM contacts, signaling molecules) differ dramatically between neurons in cell culture and new-born neurons in the dentate gyrus. Since the maturation of neurons is highly influenced by the input they receive, a direct comparison of the phenotypes (neuronal morphology, survival) between *in vitro* and *in vivo* conditions should be avoided. We

included a discussion along these lines in the discussion section of the manuscript on page 20, lines 469-491.

7. The number of cells for single cell expression analysis is low which affects the experimental power. The authors admitted to not having found any mature neurons in their samples which is very likely due to loss of elongated neurons during cell isolation from the tissue. Instead of isolating whole cells for single cell RNA-seq, the authors could attempt to prepare nuclei from brains and perform single nuclei RNA-seq. If it is not possible to get GFP-positive nuclei due to cytoplasmic location of the reporter, it may be possible to sort for BrdU, followed by NeuN and other transcription factors. Preferably the analysis would be extended to analyze several thousand nuclei as is typical for many single cell/nucleus studies now, using more brains if necessary. This would provide more insights into deficits in maturation in the KO, improve the correlation of methylation and gene expression, and allow the calling of differentially expressed genes between WT and KO.

We appreciate the comment and agree that our single-cell RNA dataset cannot be used to analyze Dnmt3a/b-dependent gene expression changes in neurons. However, as pointed out by the reviewer, the cytoplasmic location of the reporter prohibits nuclei RNA-sequencing. Sorting BrdU-positive cells is technically possible but could increase percentages of non-recombined cells in the analysis (due to the absence of a reporter for recombination). In the revised version of the manuscript, we now only used the single-cell RNA dataset to analyze potential Dnmt3a/b-dependent changes in NSPCs and early postmitotic precursor cells (now presented in Fig. 4F-J).

To gain insight into Dnmt3a/b-dependent changes in neuronal gene expression, we isolated *in vitro* differentiated neurons and performed gene expression analysis on gene candidates that were hypomethylated in KO neurons. We detected reduced mRNA levels of hypomethylated neuronal genes in Dnmt3a/b-KO neurons but not in NPCs (Fig. 3H and Fig. EV2G; main text page 11, lines 235-240). Our new results suggested that *de novo* DNA methyltransferases mediate activation of those neuronal genes during neurogenesis, which is consistent with the observed delay in neuronal maturation in Dnmt3a/b-KO mice *in vivo*.

8. The analysis/representation of single-cell data needs to be improved. First, basic QC metrics for single cell analysis must be given (mean number of reads/genes per cell, number of genes detected, % of sequencing saturation, fraction of reads mapping to mitochondrial gene and transcriptome etc, and a plot showing which cells were derived from which sample).

We detected an average of 71914.12 ± 3149.22 (mean ± SEM) reads per cell and 2824 ± 42.13 (mean ± SEM) genes per cell. As noted in Methods, the raw counts were processed at the transcript level and then collated to ENSEMBL genes. We detected a total of 35411 genes before filtering. At a threshold of 10 reads, we detected a total of 25444 genes.

The expression of mitochondrial genes as a fraction of the total transcriptome was in the range of 0.12-9.9 % with a mean of 3.8 ± 0.088 % (mean ± SEM). We employed an upper threshold of 10 % mitochondrial gene expression to filter out extreme values (potentially arising from damaged cells).

We included these descriptions in the methods section (page 33) and provide representative plots in Figures R2-R4 below.

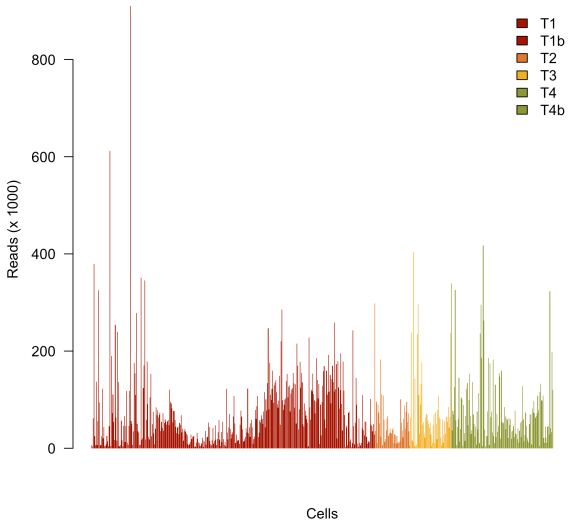

Figure R2: Numbers of reads per cell.

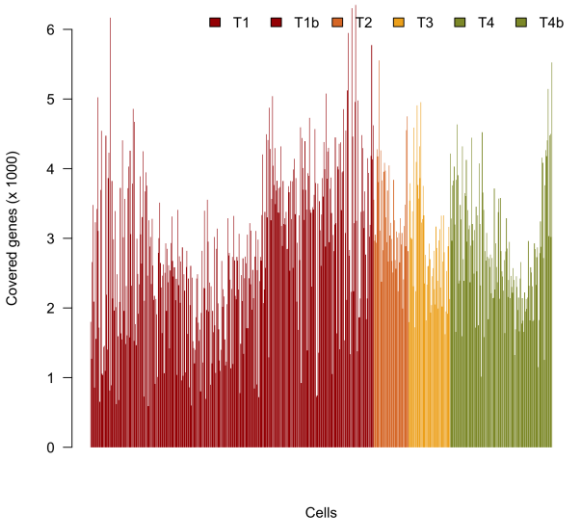

Figure R3: Numbers of genes covered per cell.

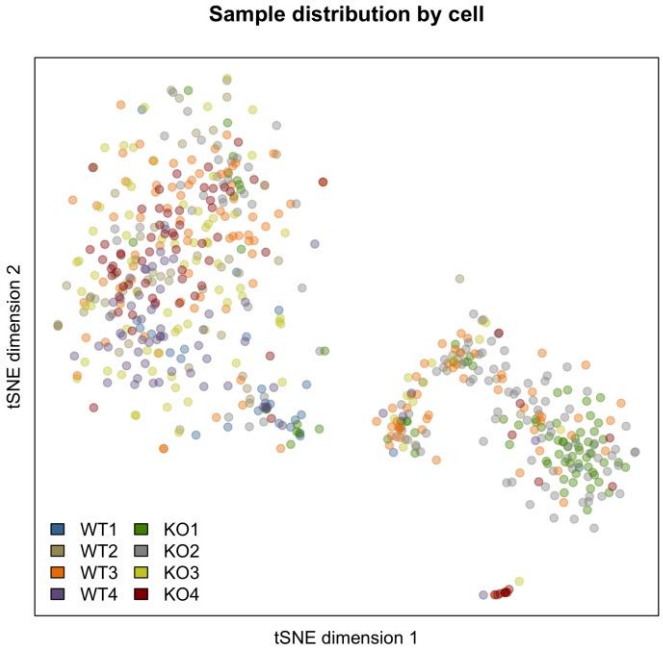

Figure R4: Plot showing which cells were derived from which sample.

Furthermore, from how many animals were these single cells obtained?

As described in the Figure legend the cells were obtained from 4 mice per genotype.

The authors should also represent the t-SNE colored by individuals and genotype.

We present the requested t-SNE plots in Figure 4H and Figure R4.

A legend for the cluster identity should be included in Fig 5A. Alternatively, and because the number of clusters is limited, the authors could replace "T1-4b" by cluster names (in Fig 5A-D).

We added a legend for the cluster identity as requested (now Fig. 4F). The clusters were named in anology of the neurogenic lineage of the hippocampus as Type-1 cells (T1), Type 2-cells (T2), Type 3-cells (T3).

The genes in Fig 5B should be reorganized vertically to match with developmental dynamics (from NSCs to immature neurons from top to bottom).

The gene order has been adjusted (now Fig. 4G).

The log fold change in Fig 5D and 5E must be specified.

The data presented in these plots was normalised so that the range of expression values in each row are scaled from -1 to 1. Thus the scale bars do not refer to absolute expression changes. Plots with absolute values are dominated by highly-expressed genes and obscure within-gene effects for lower-

expressed genes. It was not the aim of this figure to compare the expression levels *between genes* rather than *within genes* to show the change over the neurogenic trajectory.

A note has been made to the Figure legend. The Panel that was presented in 5E oft he original manuscript was removed.

9. How many genes in total are detected in both the single cell expression analysis and the DNA methylation data? About 100 genes are shown in Fig 5D, but it is not clear if these are only the de novo methylated genes with expression changes or all the de novo methylated genes.

In total, 105 *de novo* methylated genes were covered by the single-cell RNA sequencing experiment. Figure 5D (now Fig. 4J) depicts the expression change of all covered *de novo* methylated genes not just the ones with expression change. We clarified this point in the Figure legend.

10. The correlation of transcriptomic data from a limited number of cells with DNA methylation data from a method only covering a fraction of the genes is difficult and does not allow one to draw general conclusions about the effects of de novo methylation on neuronal gene expression. A method for whole genome coverage of DNA methylation should be used, e.g. WGBS to include more genes for the analysis and to allow investigation of CpH methylation changes.

We agree with the reviewer that the presented experiments do not allow claims about genome-wide effects of DNA methylation on gene expression. We carefully revised the manuscript to make sure that we did not include any such claims. We also re-wrote the manuscript to clarify that the main goal of the study was to provide a functional characterization of *de novo* DNA methyltransferases at the cellular level and not a resource of the genome-wide relationship between DNA methylation and gene expression changes during adult neurogenesis.

11. Very little analysis of the ENR-induced DNA methylation changes in the different populations was undertaken. For example, what genomic features are they located in? What genes are they associated with? Are they associated with regulatory elements, and if so, are there particular associated enriched TF motifs?

We have performed additional analyses on the of ENR-induced DNA methylation changes and included the results in Figure EV4 of the revised manuscript. The specific CpGs and corresponding genes that changed methylation in the different cell populations are presented in Table EV1. The enriched transcription factor binding motifs are reported in Table EV3. However, we decided to not further describe these results in the main text, because we feel that they would destract the reader from the main message of the manuscript.

12. The authors show that KO mice showed significantly fewer c-Fos-positive cells in the dentate gyrus and CA3 of the hippocampus after stimulation in ENR (Fig. 6D-F). It would be interesting to know if this decrease of c-FOS staining is due to a cellular autonomous mechanism (only newly generated neurons are less activated during ENR), or if the KO of Dnmts in NSCs has a more systemic effect on DG activity. To assess this, the authors should look at the double staining of BrdU + cFos to understand whether the observed decrease of c-FOS staining is only due to a lower activation of new born neurons or a more general reduced activity of the DG.

To address this question, we have analyzed c-Fos expression in adult-born (BrdU/NeuN-positive) neurons and further characterized changes in hippocampal activation patterns. These new data suggest that the difference in numbers of c-Fos-positive cells in the dentate gyrus of Dnmt3a/b-KO mice is not a results of the reduced activation of adult-born neurons themselves but rather due to their increased inhibitory output onto the hippocampal circuitry. We have included these new findings in Fig. 6F-I and describe them in the main text on page 15, lines 349-376.

Minor concerns:

- Are the percentages of CpGs that change methylation state listed on pages 5 and 9 the fraction of all sufficiently covered CpGs (based on threshold criteria outlined in methods), or all CpGs in the genome? It would be helpful to clarify this in the main text.

The depicted percentages of CpGs that changed methylation are the fraction of all sufficiently covered CpGs. We clarified this point in the figure legends and the main text.

- Fig 1C : the legend on the Y axis is missing.

We have corrected this mistake. Thanks for pointing it out.

- Fig 1F: a legend for the size of the dots should be added (I guess it represents the number of genes per GO term).

We have added legends for the dot sizes in Fig. 1F and Fig. 3G.

- Fig S1H shows the dynamics of markers across neurogenesis stages (NSCs, IPCs, Neurons). The represented markers are all NSCs markers that are downregulated during neurogenesis. It would be good to include other markers that are upregulated during neurogenesis (such as Calb2, Rbfox3...for which the data is only presented regarding DCX expression - Fig S1G).

We appreciate the comment, but believe that the presented data are sufficient to demonstrate that our isolated cell populations represent NSPCs, IPCs and neurons. Additionally, as mentioned in a previous comment, the numbers of Nestin-positive NSPCs that can be isolated from each adult hippocampus is very limited (1,500 cells per mouse), similarly limited is the population of IPCs (3000 cells per mouse). The quantitative PCR results depicted in Figure S1H are pools of 5 mice per NSPC sample and 2 mice per IPC sample. Repeating this experiment for neuronal markers (with an n = 3), would mean we would need to sacrifice 24 additional mice. Given that the marker that we used for isolation of neurons has been demonstrated to be specific to neurons before (Er *et al*, 2015), and given that it labels a percentage of cells which is expected for granule neurons of the dentate gyrus (Figure S1D), and given that we demonstrated the expected downregulation of NSPC markers (Figure S1H), we feel the supporting evidence of the neuronal population is sufficient and therefore cannot justify sacrificing the required number of mice to perform additional PCRs for neuronal markers.

- Fig S1G: why do the different genes have a different number of replicates?

Similarly as mentioned in the comment before, getting sufficient numbers of cells for qPCRs from subpopulations of DCX-positive cells is challenging and requires pooling of many mice. We did not remove any replicates from this analysis, but had performed one PCR from a sample for which we had obtained fewer cell numbers from FACS. Because the aim was to exlude the neuronal population, we performed this PCR only with neuronal markers. Since the difference in expression between the clusters was very clear, we did not repeat the cell sorting for the third replicate for stem cell markers.

Also, given the low number of replicates (2 to 3), the use of a parametric test (such as ANOVA) should be avoided and non-parametric tests should be used.

Non-parametric tests have now been used for all qPCR analyses in the paper.

- How were the methylation levels shown in Fig S2 C and D calculated? Was it all methylated CpG (or CpH) basecalls divided by total basecalls at CpG (or CpH) positions? Rather than plotting the median methylation level, which is challenging to interpret, it would be more useful to plot the distributions.

To determine global CpG and CpH methylation levels, we had calculated the median methylation levels over all covered CpGs or CpHs in one sample and compared the medians between groups. After careful consideration of the reviewer feedback regarding the sparsity of the RRBS data, we decided to remove this analysis from the manuscript, as the data only covered a subset of cytosines in the genome and do not give global information. We added statements about this limitation of our dataset in the results and discussion sections.

- Typo in Fig 3E label.

We corrected the typo.

- How have the neuronal enhancers and super-enhancers used here been defined? Please provide a table as supplementary data. Which are these super enhancers and why are they neuronal related? Some more explanation is needed.

Enhancers were retrieved from the Ensembl Regulatory Build (Zerbino *et al*, 2015), which contains functional annotation of regulatory regions based on publically available CHIP-seq data from transcription factor binding, chromatin structure, epigenomic patterns, and others. Super-enhancers were retrieved from the Super-Enhancer Archive SEA (Chen *et al*, 2020), with neuronal super-enhancers corresponding to regions derived from mouse cortex and cerebellum. We clarified these points in the methods section and added the references for each database in the main text.

- Fig 4C is confusing, and could be removed or converted to a landscape orientation. Also time points should be in weeks.

Fig 4C was simplified and converted to landscape orientation. The time point label was added. This panel can be found in Fig. 4E of the revised manuscript.

- Does Fig 4E show WT or KO mouse?

All representative images are from WT mice unless stated otherwise. We added this information to the methods section.

- Fig 4E: small squares should be added to the overlaid picture to identify cells that were magnified on the bottom panel.

Small squares indicating magnified cells have been added.

- Fig 4G: the p-value from the 2-way ANOVA indicates a significant effect. However, the different p-values for the 2 main effects (Distance to soma and Genotype) must be reported. Indeed, it seems that the reported p-value is driven by the effect of the Distance to soma and not by the Genotype. Did the authors forget to include the results of the post-hoc tests?

The *p*-value depicted in Figure 5G (previously 4G) corresponds to the genotype effect from two-way ANOVA. We have clarified this point in the Figure legend and included the effect of distance to soma and further statistical details. We also labeled significant differences at individual distances to soma with asterisks (post-hoc analysis).

- Fig 6 C: Representative photomicrographs of the STD conditions (WT and KO) must be included.

The requested photomicrographs were included in Figure EV4I.

- Fig 6 E-F: Given the low number of replicates (5 max per condition), parametric tests such as t-test cannot be used. A non-parametric test must be used instead.

Non-parametric tests should be used when the population that the samples are drawing from is not normally distributed. This is not the case when analyzing counts of cell numbers such as c-Fos-positive cells as presented in Fig. 6.

For the quantification of adult neurogensis counts, we have previously performed a large-scale study using two groups of 40 mice (STD and ENR housed; Körholz et al, 2018), in which we had analyzed different count data for adult neurogenesis (numbers of BrdU-positive cells, numbers of BrdU/NeuN-positive cells, percentages of BrdU/NeuN-positive cells, percentages of BrdU/S100b-positive cells). All these measures showed normal distributions ($p > 0.05$ with Shapiro-Wilk test using 40 mice) in both groups. This makes us confident that we are drawing from a population with normal distribution when analyzing count data for adult hippocampal neurogenesis.

However, for evaluation by the reviewer and reader, we included the results from non-parametric tests for every presented analysis in Appendix Table S5.

- Fig 7: how many animals were used per group? Same comment as above, parametric tests are probably not usable (as normality cannot be tested for less than 20-30 samples per condition).

As indicated in the Figure legend, 13 WT mice and 23 KO mice were used for water maze testing. The difference in group size was not selected for, but had occured by chance as a result of the breeding (but an increased rate of KO mice was not generally observed throughout breeding).

We tested the KO group for normality of the water maze data and found that they were indeed not normally distributed (Shapiro-Wilk test: $p < 0.05$). We therefore changed the analysis to non-parametric tests as suggested by the reviewer.

We also changed the analysis of the behavioral data in Figure EV5 to non-parametric tests, in accordance to the distributions and analyses published in (Körholz et al, 2018), where we had found non-normal distributions for those measures with group sizes of n = 40.

- Fig S5: Could a panel similar to D be added for experiments presented in B-C?

The corresponding panel is presented in Figure 4E. A note refering to Figure 4E has been added to the Figure legend (now Fig. EV3B-C).

The GFP panel for the astrocytes in panel C must be included, and small squares in the main overlay must be added to highlight magnified cells.

Squares have been added to the merged picture. The Gfp panel was omitted for the astrocyte, because the astrocyte was Gfp-negative (we now mention this in the Figure legend). Because of the very low percentages of Gfp-positive astrocytes, it was very difficult to get an image demonstrating both Gfp-positive NSPCs and Gfp-positive NSPCs. The image we present in Fig. S5 (now Fig. EV3C) however does demonstrate the quality of the antibody staining, which should be the main point of such representative images.

In the legend, "D" and "G" letters must be put in bold.

Corrected.

- Fig S8F: The discrimination index indicates that neither the WT nor the KO animals spent more time exploring the new object compared to the old one. This is not what is expected for an object recognition test and could indicate a lack of memory for both genotypes. Please check and comment whether this lack of preference for the novel object is observed for both groups.

For the object exploration test, we had used a very similar setup as in our previous studies (Körholz *et al*, 2018; Zocher *et al*, 2020). Already in Körholz *et al*, 2018 we had observed that C57BL6 mice exhibit a discrimination index of 0 and it was only after environmental enrichment that they showed a slight increase to a ratio of 0.4. Therefore, we had later used this test rather as a measure of object exploration than of recognition memory. We do not think that a discrimination ratio of 0 suggests that the Dnmt3a/b WT mice used in the present study exhibit a lack of recognition memory, but appreciate that the setup might not be ideal to measure recognition memory. We therefore removed the Figure panel with the discrimination index together with claims about recognition memory, and present the object exploration data only as a measure of exploratory activity of the mice.

- Page 5, l. 14: the reference for Boyle et al. should be replaced by the appropriate number. Similar changes have to be made on page 19, l. 2 and 22).

Corrected.

- Page 6, l. 3: the full list of enriched motifs for de novo methylated CpGs should be presented in a supplementary table.

The full lists of enriched motifs for the datasets analyzed in Figure 1, Figure 3 and Figure EV6 are presented in Table EV3.

- Page 7, l. 7: the word "synaptic plasticity" should be rephrased as there is nothing in the data presented that links these genes to synaptic plasticity (which is a specific and well documented physiological phenomenon).

We removed the term „synaptic plasticity“.

- Page 10, l. 15 and l. 17: Shouldn't it be Fig. S5 and not Fig. S6?

Corrected. Corresponds to Fig. EV3 in the revised manuscript.

- Page 20: for the drugs used, please add the catalogue number and the concentration to which they were dissolved (prior to injection).

We included this information in the methods section.

- Page 22, last line: the last word of the line ("and") must be deleted.

Corrected.

- Page 24, l. 4: were the brains transcardially perfused with sodium chloride only? In general, for immunohistochemistry, animals are perfused with PFA following saline perfusion in order to directly, immediately and completely fix brain tissue. Please check and clarify.

The brains were perfused with sodium chloride, removed from the skull, bisected and postfixed with PFA overnight as described in the methods section. Postfixation in PFA is sufficient to fix brains. We have successfully used this protocol in numerous previous studies; e. g. (Zocher *et al*, 2020; Adusumilli *et al*, 2020; Körholz *et al*, 2018; Overall *et al*, 2013).

- Page 25, please include to the table the references and dilutions of the secondary antibodies.

We now present this information for the secondary antibodies in Appendix Table S4.

- Page 27, l. 18: The size of the open field is probably not 50 cm2 but 50cm*50cm.

This mistake was corrected. Thanks for pointing it out.

Referee #3:

The manuscript interrogates the role of de novo DNA methyltransferases Dnmt3a and Dnmt3b in adult neurogenesis and brain function using elegant mouse genetics, DNA methylome mapping and behavioral studies. In general the work is done with rigor and presented with clarity.
While the manuscript provides a careful dissection of the role of Dnmt3a/b in neurogenesis and hippocampal function, the major weakness is that it does not provide molecular insights by identifying genes regulated by DNA methylation in neurogenesis. Therefore the manuscript brings little conceptual advances compared to previous work that already demonstrated the importance of Dnmt3 enzymes for mouse neurogenesis.

Major points:

-The overall originality of the study is uncertain given that several previous studies combined Dnmt3a-2lox mice with Nestin-Cre or Nestin-CreERT2 for conditional deletion in neural progenitor cells. The authors should better explain how their study complements previous work and addresses novel unsolved questions.

We are only aware of the studies by (Nguyen *et al*, 2007) and (Odell *et al*, 2020) who used Nestin-Cre/Dnmt3a-2lox mice or Nestin-CreRT2/Dnmt3a-2lox mice to ablate *Dnmt3a* in embryonic NSPCs. Both studies analyzed the role of Dnmt3a during embryonic and early postnatal brain development. Other studies like (Stroud *et al*, 2017, 2020) had used Nestin-Cre/Dnmt3a-2lox mice to characterize effects on transcription factor binding in the early postnatal brain but did not present cellular or behavioral characterizations. However, to the best of our knowledge, no study had examined the role of Dnmt3a (or 3b) during adult neurogenesis in the hippocampus before. Importantly, adult hippocampal neurogenesis is not just a continuation of brain development, and adult hippocampal NSPCs are molecularly and functionally distinct from embryonic NSPCs. No previous study had reported DNA methylation profiles of adult hippocampal NSPCs before, and no study had analyzed roles of Dnmt3a (or 3b) during adult hippocampal NSPC differentiation. By demonstrating that Dnmt3a/b control the morphological and functional maturation of new-born neurons in the hippocampus (but not their neuronal fate or survival), we have answered a previously unresolved question.

Moreover, while previous studies had analyzed the cellular consequences of deleting *de novo* DNA methyltransferases during NSPC differentiation (Ziller *et al*, 2018; Wu *et al*, 2010, 2012), all of these studies were limited to *in vitro* analyses. Given that neuronal differentiation/maturation is highly dependent on extracellular input, we want to highlight that a particular strength of our study is the amount of *in vivo* phenotypic characterization it provides (from DNA methylation/ RNA sequencing to cell stage-specific cellular characterizations to circuit analyses and cognitive testing).

To better emphasize the novel aspects of our work, we rephrased the abstract, introduction and discussion of the manuscript.

-More information is needed about the quality of the RRBS datasets: sequencing depth, number of covered CpGs, number of replicates, correlation between replicates, etc...Without this information it is impossible to evaluate the quality of the datasets on which are based the manuscript's conclusions.

This information is now presented for all datasets in Appendix Table S1. We also revised the manuscript to include the exact replicate number for each analysis in the respective Figure legends.

In terms of correlation between replicates: because the large number of samples would lead to many pages of correlation plots, we here provide an example for the first six samples presented in Table S1.

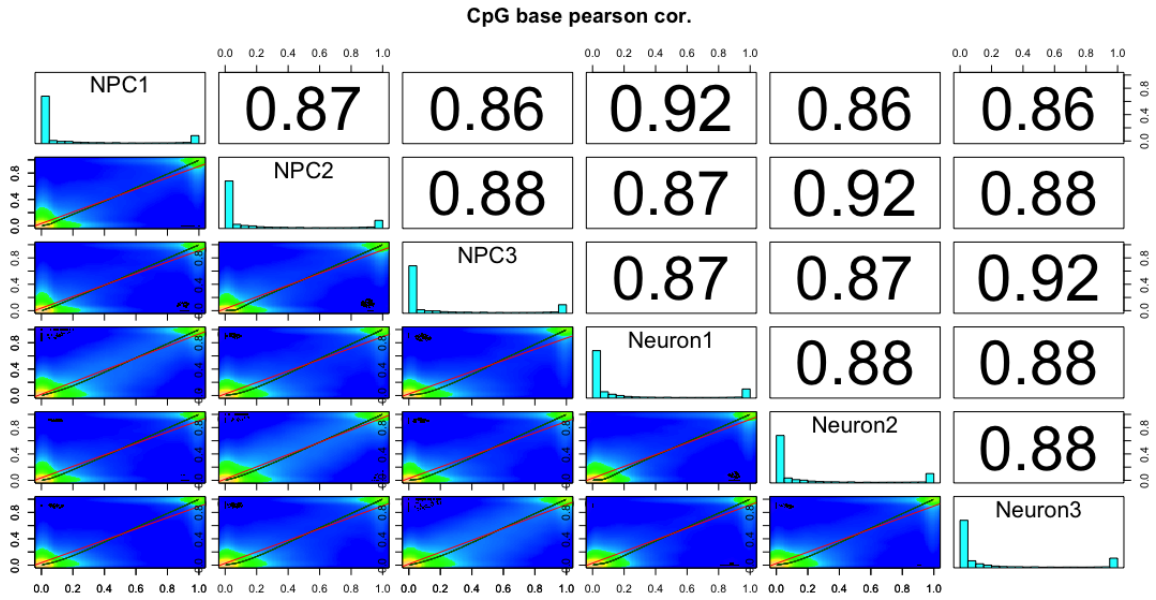

Figure R5: Correlation between replicates within one RRBS sequencing experiment.

-I am a bit skeptical about the motif analysis in Figure 1D because it looks like it simply reflects the enrichment of the CG dinucleotide in sites of methylation.

The motif analysis was performed using all *de novo* methylated CpGs as a query and all CpGs covered by RRBS as background list. The result was a significant enrichment of Mecp2 in *de novo* methylated CpGs over background CpGs.

-It is important to know whether there are global changes in CpH and CpG methylation during neurogenesis. In contrast to what is claimed in the text, Figure S2D does not seem to address this because it shows methylation of an unknown set of 765 CpGs. Average CpG methylation scores of all CpGs should be shown.

In the previous version of the manuscript, we had calculated global CpG and CpH methylation changes based on the cytosine positions that were covered by RRBS in all samples of all datasets. We agree that analyzing average methylation only for such a small subset of CpGs is difficult to interpret and that the limited coverage of the RRBS data does not allow making claims about global/ genome-wide methylation changes. We therefore removed those plots and state in the result and discussion section the limitations of not providing global DNA methylation changes.

-In Figure 3, RRBS should also be performed in in vitro NSPCs before neuronal differentiation. This would allow addressing two important questions:1) to what extent do changes in methylation during

in vitro neuronal differentiation recapitulate the in vivo patterns seen in Figure 1? 2) Do hypomethylated CpGs in Dnmt3a/b KO neurons reflect failure in de novo or maintenance methylation?

As suggested by the reviewer, we have performed RRBS on *in vitro* NPCs (WT and KO) and included these data in the revised manuscript (Figure EV3 and main text page 10, lines 211-223). To address the first question, we calculated differentially methylated CpGs between *in vitro* WT NPCs and WT neurons and compared them with the methylation changes observed *in vivo*. We identified 1,551 CpGs (0.50 % of CpGs covered by RRBS) that changed methylation during neuronal differentiation *in vitro* in WT cultures (Figure EV3D-F). This corresponded to a smaller fraction of differentially methylated CpGs compared to what we had detected *in vivo*. Additionally, we found only little overlap of the CpGs that change DNA methylation during neurogenesis *in vivo* and *in vitro* (Fig. EV3D-F). Possible explanations for this discrepancy between *in vivo* and *in vitro* methylation changes include potential differences in developmental stages between *in vivo* NSPCs and *in vitro* NPCs, different contributions of maintenance methylation *in vivo* and *in vitro* and consequences of extracellular signals, which are very different *in vivo* versus *in vitro*, and are known to influence DNA methylation patterns. We included a discussion along these lines on page 20, lines 469-492.

To answer the second question raised by the reviewer, we determined the fraction of hypo/hypermethylated CpGs in *Dnmt3a/b*-KO neurons that was already hypo/hypermethylated in *Dnmt3a/b*-KO NPCs. We found that 60.9 % of the hypomethylated CpGs and 13.9 % of the hypermethylated CpGs preexisted at the NPC stage, suggesting that Dnmt3a/b contribute to maintenance methylation in NPCs *in vitro*. However, whether a similar pattern would be observed *in vivo* remains unknown. While *in vivo* hippocampal NSPCs go through 2-4 rounds of divisions before they terminally differentiate into neurons (Pilz *et al*, 2018; Bottes *et al*, 2021), *in vitro* NPCs continuously proliferate and underwent at least 20 rounds of divisions before analysis as every culture was clonally expanded. Given that the role of Dnmt3a/b in maintenance methylation is likely an indirect one (by compensating infidelity of Dnmt1), their effect on maintenance methylation is likely amplified *in vitro* compared to *in vivo*. We present these data in Figure EV3A-C, the main text (page 10, lines 211-220) and the discussion section (page 20, lines 468-490).

We want to emphasize that although we detected these potential differences between DNA methylation changes *in vivo* versus *in vitro*, this does not comprise the conclusions of our study. Most of our conclusions of the phenotypic consequences of Dnmt3a/b are drawn from *in vivo* experiments. The main point of the *in vitro* experiments in the original manuscript was to validate the effects of *Dnmt3a/b*-KO at the level of DNA methylation, as this was technically not feasable *in vivo*.

-The central question of the work is whether de novo methylation events are required to regulate gene transcription events during neurogenesis. Quite disappointingly, the authors found little transcriptional changes in conditional KO animals by single cell RNA-seq and are thus unable to relate aberrant DNA methylation to perturbed gene expression in neurogenesis. However I don't find that this is addressed in a satisfactory manner because the authors do not perform methylome and transcriptome analyses in the same cellular samples. Why not doing RNA-seq on in vitro differentiated neurons to directly relate methylation and gene expression in the same cells?

First, we want to emphasize that understanding how DNA methylation regulates gene transcription was not the central question of this work. Our main goal was to provide a phenotypic analysis of the role of *de novo* DNA methyltransferases for adult hippocampal NSPC proliferation, fate specification, neuron survival, neuron maturation as well as to investigate how deleting Dnmt3a/b specifically in adult NSPCs affects hippocampal circuits and function, as these points had been unknown. We re-wrote the manuscript to clarify the main objectives of our study and highlight in the discussion that a more detailed molecular characterization (e. g. identifying and manipulating specific genes) should be performed in future studies.

However, we do appreciate the comment of the reviewer as we were also disappointed with the small gene expression differences detected between WT and KO mice in the single-cell RNAseq experiment. At the same time, we feel that the finding of minor Dnmt3a/b-dependent transcription changes during progenitor stages of adult hippocampal neurogenesis is interesting and indeed consistent with the results from our phentoypic analyses, which showed little effects of *Dnmt3a/b*-KO on NSPCs proliferation, neurogenic activity and long-term maintenance . We agree with the reviewer that comparing DNA methylation and transcription data in different cell populations is not ideal. However, since the *Dnmt3a/b*-KO mice have neither a *Nestin*::Gfp nor a *Dcx*::Gfp reporter (which would have allowed for direct comparison of transcription with RRBS data), and since performing single-cell methylation sequencing comparable to the cell populations of the single-cell RNAseq would have likely not yielded better genome coverage, we feel that analyzing the different cell populations as we did was, at that time, the best solution for studying *in vivo* adult neurogenesis.

To further address the reviewers concern, we have now performed qRT-PCRs for the gene candidates for which we had found Dnmt3a/b-dependent hypomethylation in neurons *in vitro*. These new results suggested that loss of methylation at those gene candidates resulted in their transcriptional down-regulation in neurons (Fig. 3H), which is consistent with the predominant up-regulation of *de novo* methylated genes found in the single-cell RNAseq experiment. However, we think that performing the suggested RNA sequencing on *in vitro* neurons or (*in vitro* NPCs) would not improve our manuscript, given that understanding *in vitro* neuronal differentiation was not our goal. Additionally, a number of previous studies have already characterized the relationship between DNA methylation and transcription during *in vitro* neuronal differentiation on a genome-wide scale (see for instance Mohn *et al*, 2008; Ziller *et al*, 2018; Xie *et al*, 2013; Luo *et al*, 2016).

-Although RRBS provides accurate and quantitative methylation calls, it provides very poor coverage of gene bodies and enhancers, thus limiting data interpretation. WGBS would certainly be much more informative. I acknowledge that WGBS is costly and requires more DNA but it could be done on WT and KO in vitro neurons.

We fully agree that WGBS would provide better genomic coverage, particularly of gene bodies and enhancers, and would provide more information. However, as appreciated by the reviewer, the amount of DNA obtained from NSPCs or lPCs *in vivo* would not be sufficient for high coverage WGBS (we obtain 1,500 NSPCs and 3,000 lPCs per mouse). Moreover, the major motivation of this study was to perform *in vivo* functional characterization of *de novo* DNA methylation at the cellular level. The point of performing RRBS on *in vitro* neurons was mainly to validate that deletion of Dnmt3a/b resulted in the expected loss of DNA methylation in neurons (which it did) and not to characterize DNA methylation changes during *in vitro* neuronal differentiation. The latter has already been done

in previous studies as pointed out by the reviewer earlier (see for instance Mohn *et al*, 2008; Ziller *et al*, 2018; Xie *et al*, 2013; Luo *et al*, 2016). Therefore, we believe that performing WGBS on *in vitro* differentiated cells would not improve our manuscript at this stage.

To address the reviewers concern, we discuss the limitations of RRBS data compared to WGBS in the result and discussion section of the revised manuscript (page 18, lines 424-436).

-Explain what is shown on the y axis in Figure 1C.

The label of the y axis has been added.

-Please explain with more details what annotations were used to define enhancers in the study.

Enhancer locations were retrieved from the Ensembl Regulatory Build (Zerbino *et al*, 2015), which contains functional annotation of regulatory regions based on publically available CHIP-seq data from transcription factor binding, chromatin structure, epigenomic patterns, and others. Super-enhancers were retreived from the Super-Enhancer Archive SEA (Chen *et al*, 2020), with neuronal super-enhancers corresponding to regions derived from mouse cortex and cerebellum. We clarified these points in the methods (page 32, lines 752-756) and added the references for each database in the results section (page 9, line 202).

-The sequencing data should be made accessible in GEO at time of submission for review purposes.

Sequencing data are now accessible at GEO (accession number GSE167955).

References related to point-by-point responses

Adusumilli VS, Walker TL, Overall RW, Klatt GM, Zeidan SA, Zocher S, Kirova DG, Ntitsias K, Fischer TJ, Sykes AM, *et al* (2021) ROS Dynamics Delineate Functional States of Hippocampal Neural Stem Cells and Link to Their Activity-Dependent Exit from Quiescence. *Cell Stem Cell* 28: 300–314

Bottes S, Jaeger BN, Pilz GA, Jörg DJ, Cole JD, Kruse M, Harris L, Korobeynyk VI, Mallona I, Helmchen F, *et al* (2021) Long-term self-renewing stem cells in the adult mouse hippocampus identified by intravital imaging. *Nat Neurosci* 24: 225–233

Chen C, Zhou D, Gu Y, Wang C, Zhang M, Lin X, Xing J, Wang H & Zhang Y (2020) SEA version 3.0: A comprehensive extension and update of the Super-Enhancer archive. *Nucleic Acids Res* 48: D198–D203

Er JC, Leong C, Teoh CL, Yuan Q, Merchant P, Dunn M, Sulzer D, Sames D, Bhinge A, Kim D, *et al* (2015) NeuO: a Fluorescent Chemical Probe for Live Neuron Labeling. *Angew Chemie* 127: 2472–2476

Fabregat A, Jupe S, Matthews L, Sidiropoulos K, Gillespie M, Garapati P, Haw R, Jassal B, Korninger F, May B, *et al* (2018) The Reactome Pathway Knowledgebase. *Nucleic Acid Res* 46: 649–655

Körholz JC, Zocher S, Grzyb AN, Morisse B, Poetzsch A, Ehret F, Schmied C & Kempermann G (2018) Selective increases in inter-individual variability in response to environmental enrichment in female mice. *Elife* 7

Luo C, Lancaster MA, Castanon R, Nery JR, Knoblich JA & Ecker JR (2016) Cerebral Organoids Recapitulate Epigenomic Signatures of the Human Fetal Brain. *Cell Rep* 17: 3369–3384

Mohn F, Weber M, Rebhan M, Roloff TC, Richter J, Stadler MB, Bibel M & Schübeler D (2008) Lineage-Specific Polycomb Targets and De Novo DNA Methylation Define Restriction and Potential of Neuronal Progenitors. *Mol Cell* 30: 755–766

Nguyen S, Meletis K, Fu D, Jhaveri S & Jaenisch R (2007) Ablation of de novo DNA methyltransferase Dnmt3a in the nervous system leads to neuromuscular defects and shortened lifespan. *Dev Dyn* 236: 1663–1676

Noack F, Pataskar A, Schneider M, Buchholz F, Tiwari VK & Calegari F (2019) Assessment and site-specific manipulation of DNA (hydroxy-)methylation during mouse corticogenesis. *Life Sci Alliance* 2: 1–15

Odell SC, Taki F, Klein SL, Chen RJ, Levine OB, Skelly MJ, Nabila A, Brindley E, Toth JG, Dündar F, *et al* (2020) Epigenomically Bistable Regions across Neuron- Specific Genes Govern Neuron Eligibility to a Coding Ensemble in the Hippocampus. *Cell Rep* 31: 107789

Overall RW, Walker TL, Leiter O, Lenke S, Ruhwald S & Kempermann G (2013) Delayed and Transient Increase of Adult Hippocampal Neurogenesis by Physical Exercise in DBA/2 Mice. *PLoS One* 8: e83797

Pilz GA, Bottes S, Betizeau M, Jörg DJ, Carta S, Simons BD, Helmchen F & Jessberger S (2018) Live imaging of neurogenesis in the adult mouse hippocampus. *Science (80-)* 359: 658–662

Stroud H, Su SC, Hrvatin S, Kinde B, Gabel HW, Greenberg ME, Stroud H, Su SC, Hrvatin S, Greben AW, *et al* (2017) Early-Life Gene Expression in Neurons Modulates Lasting Epigenetic States. *Cell* 171: 1151–1154

Stroud H, Yang MG, Tsitohay YN, Davis CP, Sherman MA, Hrvatin S, Ling E & Greenberg ME (2020) An Activity-Mediated Transition in Transcription in Early Postnatal Neurons. *Neuron*: 1–17

Wu H, Coskun V, Tao J, Xie W, Ge W, Yoshikawa K, Li E, Zhang Y & Sun YE (2010) Dnmt3a-Dependent Nonpromoter DNA Methylation Facilitates Transcription of Neurogenic Genes. *Science (80-)* 329: 444–449

Wu Z, Huang K, Yu J, Le T, Namihira M, Liu Y, Zhang J, Xue Z, Cheng L & Fan G (2012) Dnmt3a regulates both proliferation and differentiation of mouse neural stem cells. *J Neurosci Res* 90: 1883–1891

Xie W, Schultz MD, Lister R, Hou Z, Rajagopal N, Ray P, Whitaker JW, Tian S, Hawkins RD, Leung D, *et al* (2013) Epigenomic analysis of multilineage differentiation of human embryonic stem cells. *Cell* 153: 1134–1148

Yu G & He Q (2016) Molecular BioSystems ReactomePA : an R / Bioconductor package for reactome pathway analysis and visualization. *Mol Biosyst* 12: 477–479

Zerbino DR, Wilder SP, Johnson N, Juettemann T & Flicek PR (2015) The Ensembl Regulatory Build. *Genome Biol* 16: 1–8

Ziller MJ, Edri R, Yaffe Y, Donaghey J, Pop R, Mallard W, Issner R, Gifford C a, Goren A, Xing J, *et al* (2015) Dissecting neural differentiation regulatory networks through epigenetic footprinting. *Nature* 518: 355–359

Ziller MJ, Ortega JA, Quinlan KA, Santos DP, Gu H, Martin EJ, Galonska C, Pop R, Maidl S, Di Pardo A, *et al* (2018) Dissecting the Functional Consequences of De Novo DNA Methylation Dynamics in Human Motor Neuron Differentiation and Physiology. *Cell Stem Cell* 22: 559–574

Zocher S, Schilling S, Grzyb AN, Adusumilli VS, Bogado Lopes J, Günther S, Overall RW, Winter Y & Kempermann G (2020) Early-life environmental enrichment generates persistent individualized behavior in mice. *Sci Adv* 6: eabb1478

1st Revision - Editorial Decision 15th Jun 2021

Dear Sara,

I am sorry for the delay in getting back to you with a decision, but I have now received the needed input from both referees #2 and 3 on the in vivo DNA methylation data shown in Figure 1. They both raise concerns regarding the quality of the data set. I realise that it is not your intention for the field to use this dataset as a resource, but nevertheless if the quality is not good because of low coverage then better to remove the dataset than keeping it in. I have provided comments from the referees on the provided point-by-point response below the referee comments.

Even without this dataset, I still see the value of the manuscript and would move forward with the manuscript, but with removing the in vivo DNA methylation data shown in Figure 1.

Regarding point #2 raised by referee #3 - I agree best not to include figure R1 in the final MS.

It will take some re-organisation of the MS. Will you send me an outline of what the new figure 1 will look like and what has changed. Please also address the other raised points.

When you submit the revised version will you also take care of the following points:

- "Declaration of Interests" should be referred to as Conflict of Interest

- The author contributions are missing for Christine Steinhauer

- Figure callouts are missing for Fig 7G, Fig EV3G & Fig EV5.

- We include a synopsis of the paper (see http://emboj.embopress.org/). Please provide me with a general summary statement and 3-5 bullet points that capture the key findings of the paper.

- We also need a summary figure for the synopsis. The size should be 550 wide by [200-400] high (pixels). You can also use something from the figures if that is easier.

- I have asked our publisher to do their checks on the paper. They will send me the file within the next few days and I will pass it on to you.

So let's discuss figure 1. We are almost there!

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Information is available in our Guide For Authors:
https://www.embopress.org/page/journal/14602075/authorguide

The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 13th Sep 2021.

https://emboj.msubmit.net/cgi-bin/main.plex

Referee #1:

Dynamic changes in DNA methylation have been found to significantly contribute to (neuro-) developmental processes in particular to cell-type specification and differentiation. This manuscript uses state-of-the-art genomic and transcriptomic analyses combined with histological and behavioral analyses to investigate how de novo methylation catalyzed by DNMT3a and/or DNMT3b regulates the generation of new neurons in the hippocampus of adult mice.The revised study is well-conceived. It a) provides an important resource on developmental stage-specific DNA methylation in adult hippocampal neurogenesis, b) describes for the first time the function of DNMT3a/b in adult hippocampal neurogenesis, c) uncovers an uncoupling of neuronal cell-fate decisions from de novo methylation.
The authors have addressed all my previously raised concerns.
I do have a few minor comments:
1. in lines 182-185 the authors write "...However, this difference may more likely be explained by a differential effect on survival (potentially through increased trophic support and higher number of cell contacts from astrocytes in the KO cultures) rather than by a difference in NPC potential...." There is no evidence for this claim and I would suggest to remove this sentence.
2. in lines 192-193 the authors write: "...Together, these results showed that de novo DNA methyltransferases influence numbers and maturation of the neuronal and astrocytic progeny of hippocampal NPCs in vitro...." The in vitro data shows that neurons and astrocytes in the KO have an altered morphology. THere is no evidence for altered maturation. Pleas rephrase.
3. The mechanism responsible for the altered performance in behavior tests and the altered IEG activity is still unclear. THe authors should consider(in particular in light of the observation that dnmt3a/b KO results in methylation changes in the enhancer of genes related to synapse formation) to discuss the possibility that the behavioral deficits / altered network activity could be caused by changes in the connectivity to pre/postsynaptic partners.

Referee #2:

In the revised manuscript the authors have greatly improved the study and addressed basically all of my initial comments. Where a suggested additional experiment was not performed (e.g. WGBS), the authors have clarified the significant challenge of doing this, and have suitably revised the claims and analyses and discussion to acknowledge the limitation and resolve the initial critique. The revised manuscript benefits from better and more cautious wording, more details, and clearer descriptions. Overall, this is a good study and it is reasonable that significant additional animal experiments are not performed for some minor improvements. I have only the following remaining comments below.

1. New Fig 2F-G and Fig S2D: Fig S2D should be included in the main Figure 2 as it actually shows the opposite (reduction of the fraction of astrocytes) to what picture 2G tends to show (increase of

proportion of astrocytes).

2. Fig EV3C: The square for the astrocyte is still missing. I still think that GFP panel should be added for the astrocyte, even if negative. The S100b channel is presented for NSPC (which is S100b negative), so why not doing the same for astrocyte?

Referee #3:

The author revised their manuscript in response to my comments. Some of these responses are satisfactory. For example, the authors improved the text to highlight the originality of their study (the in vivo study of Dnmt3 enzymes during adult neurogenesis) compared to previous work. They also argue that their focus is to describe the phenotype of the mice rather than provide molecular mechanisms, therefore the manuscript remains weak on investigating the effects of DNMT3a/b on neuronal gene expression.

However I still have major concerns about the DNA methylation analysis presented in the manuscript, as detailed below.

-The authors now provide details about the quality of the RRBS data and access to the GEO dataset. Opportunistically the show in their response letter acceptable correlations for RRBS datasets generated from the in vitro samples (prepared from 100 ng DNA). However, if I am not mistaken, the processed data files available from GEO indicate that the quality of in vivo RRBS datasets (mainly IPCs and NSPCs prepared from 8,000 cells) is very low. Many replicates have less than 30,000 covered CpGs and the correlations between replicates are bad. This explains that only 765 CpGs were commonly covered by RRBS in all samples in the initial version of the manuscript, while RRBS typically covers ~1 million CpGs in the mouse genome and is suitable for very low amounts of starting DNA. Again if I am not mistaken, I wonder what conclusions can be drawn from such low quality datasets in the Figure 1 and in the comparison with the in vitro datasets (Figure EV2F).

-On page 10, I do not agree with the interpretation that the data suggests a maintenance function of Dnmt3a/b. The fact that hypomethylated CpGs preexist in NPCs is not proof of a maintenance function because we don't know the initial methylation state before adaptation to in vitro culture. An example of maintenance methylation would be CpGs methylated in WT and KO NPCs and demethylated in KO neurons.

Minor comment:
-Add a color legend to Figure 3H

Referee #2 on the point-by-point by response regarding quality of the RRBS data

"I've gone over the new point-by-point response from the authors related to this issue. The inter-sample variability is clearly high for the NSPCs and IPCs, with a large range in the covered CpGs between samples (e.g. 70k vs 600k), so it is clear that some of the datasets are lower quality than would be desired, which does have significant consequences for the analyses that could be performed. Nonetheless, as the authors point out, most of the conclusions in their study are drawn from the cellular and behavioral analyses that comprise most of the results presented, rather than a

primary focus on genome scale methylome analyses, so this does only affect a portion of the study."

Referee #3 on the point-by-point by response regarding quality of the RRBS data

Point 1:
My opinion is that the in vivo RRBS data for NSPCs and IPCs is not publication quality. The very low number of covered CpGs (frequently less than 100,000), the high sequencing depth, the binary distribution of methylation (0 or 1) and the low correlation between replicates (r=0.30) all indicate that the PCR for the library amplification was biased and always amplified the same molecules (hence the binary methylation, high sequencing depth and low coverage).

Therefore, I am not confident in the in vivo methylation data presented in Figure 1. As argued by the authors, this dataset should not be viewed as a resource for genome-wide changes in DNA methylation during neurogenesis but this is how the future readers will see it and use it. My suggestion is that it should be removed from the manuscript.

Point 2:
About the second part of the letter on maintenance methylation, I agree
with the author's response. OK for replacing "maintenance" by "pre-existing", but I would not include Figure R1 in the manuscript because this represents only 1.44% of CpGs (possible technical artefacts). In fact, it rather shows that 98,56% of CpGs do not show a pattern compatible with a maintenance function, indicating that Dnmt3a/b function is mainly related to de novo methylation.

Referee #1:

Dynamic changes in DNA methylation have been found to significantly contribute to (neuro-) developmental processes in particular to cell-type specification and differentiation. This manuscript uses state-of-the-art genomic and transcriptomic analyses combined with histological and behavioral analyses to investigate how de novo methylation catalyzed by DNMT3a and/or DNMT3b regulates the generation of new neurons in the hippocampus of adult mice.The revised study is well-conceived. It a) provides an important resource on developmental stage-specific DNA methylation in adult hippocampal neurogenesis, b) describes for the first time the function of DNMT3a/b in adult hippocampal neurogenesis, c) uncovers an uncoupling of neuronal cell-fate decisions from de novo methylation.
The authors have addressed all my previously raised concerns.
I do have a few minor comments:
1. in lines 182-185 the authors write "...However, this difference may more likely be explained by a differential effect on survival (potentially through increased trophic support and higher number of cell contacts from astrocytes in the KO cultures) rather than by a difference in NPC potential...." There is no evidence for this claim and I would suggest to remove this sentence.

We agree with the referee and removed this sentence from the manuscript.

2. in lines 192-193 the authors write: "...Together, these results showed that de novo DNA methyltransferases influence numbers and maturation of the neuronal and astrocytic progeny of hippocampal NPCs in vitro...." The in vitro data shows that neurons and astrocytes in the KO have an altered morphology. THere is no evidence for altered maturation. Pleas rephrase.

We replaced the word „maturation“ with „morphology“.

3. The mechanism responsible for the altered performance in behavior tests and the altered IEG activity is still unclear. THe authors should consider(in particular in light of the observation that dnmt3a/b KO results in methylation changes in the enhancer of genes related to synapse formation) to discuss the possibility that the behavioral deficits / altered network activity could be caused by changes in the connectivity to pre/postsynaptic partners.

We have added a sentence in the discussion on page 20, lines 461-465, which reads as follows: *“The hypomethylation of genomic loci with known roles in synapse organization and the reduced numbers of spines observed in Dnmt3a/b-deficient neurons suggest alterations in pre- and/or postsynaptic connections as a potential mechanism underlying the cognitive deficits of Dnmt3a/b-KO mice.”*

Referee #2:

In the revised manuscript the authors have greatly improved the study and addressed basically all of my initial comments. Where a suggested additional experiment was not performed (e.g. WGBS), the authors have clarified the significant challenge of doing this, and have suitably revised the claims and analyses and discussion to acknowledge the limitation and resolve the initial critique. The revised

manuscript benefits from better and more cautious wording, more details, and clearer descriptions. Overall, this is a good study and it is reasonable that significant additional animal experiments are not performed for some minor improvements. I have only the following remaining comments below.

1. New Fig 2F-G and Fig S2D: Fig S2D should be included in the main Figure 2 as it actually shows the opposite (reduction of the fraction of astrocytes) to what picture 2G tends to show (increase of proportion of astrocytes).

Due to space limitations in Figure 2 and after consulting with the editor, we decided to leave Figure S2D in the Appendix.

2. Fig EV3C: The square for the astrocyte is still missing. I still think that GFP panel should be added for the astrocyte, even if negative. The S100b channel is presented for NSPC (which is S100b negative), so why not doing the same for astrocyte?

We have added the square and the Gfp panel for the astrocyte to the image presented in Figure EV3C.

Referee #3:

The author revised their manuscript in response to my comments. Some of these responses are satisfactory. For example, the authors improved the text to highlight the originality of their study (the in vivo study of Dnmt3 enzymes during adult neurogenesis) compared to previous work. They also argue that their focus is to describe the phenotype of the mice rather than provide molecular mechanisms, therefore the manuscript remains weak on investigating the effects of DNMT3a/b on neuronal gene expression.

However I still have major concerns about the DNA methylation analysis presented in the manuscript, as detailed below.

-The authors now provide details about the quality of the RRBS data and access to the GEO dataset. Opportunistically the show in their response letter acceptable correlations for RRBS datasets generated from the in vitro samples (prepared from 100 ng DNA). However, if I am not mistaken, the processed data files available from GEO indicate that the quality of in vivo RRBS datasets (mainly IPCs and NSPCs prepared from 8,000 cells) is very low. Many replicates have less than 30,000 covered CpGs and the correlations between replicates are bad. This explains that only 765 CpGs were commonly covered by RRBS in all samples in the initial version of the manuscript, while RRBS typically covers ~1 million CpGs in the mouse genome and is suitable for very low amounts of starting DNA. Again if I am not mistaken, I wonder what conclusions can be drawn from such low quality datasets in the Figure 1 and in the comparison with the in vitro datasets (Figure EV2F).

The response to this comment had been included in the previous round of review.

-On page 10, I do not agree with the interpretation that the data suggests a maintenance function of Dnmt3a/b. The fact that hypomethylated CpGs preexist in NPCs is not proof of a maintenance function because we don't know the initial methylation state before adaptation to in vitro culture. An

example of maintenance methylation would be CpGs methylated in WT and KO NPCs and demethylated in KO neurons.

The response to this comment had been included in the previous round of review.

Minor comment:
-Add a color legend to Figure 3H

A color legend was added to Figure 3H.

Referee #2 on the point-by-point response regarding quality of the RRBS data

"I've gone over the new point-by-point response from the authors related to this issue. The inter-sample variability is clearly high for the NSPCs and lPCs, with a large range in the covered CpGs between samples (e.g. 70k vs 600k), so it is clear that some of the datasets are lower quality than would be desired, which does have significant consequences for the analyses that could be performed. Nonetheless, as the authors point out, most of the conclusions in their study are drawn from the cellular and behavioral analyses that comprise most of the results presented, rather than a primary focus on genome scale methylome analyses, so this does only affect a portion of the study."

Referee #3 on the point-by-point by response regarding quality of the RRBS data

Point 1:
My opinion is that the in vivo RRBS data for NSPCs and IPCs is not publication quality. The very low number of covered CpGs (frequently less than 100,000), the high sequencing depth, the binary distribution of methylation (0 or 1) and the low correlation between replicates (r=0.30) all indicate that the PCR for the library amplification was biased and always amplified the same molecules (hence the binary methylation, high sequencing depth and low coverage).

Therefore, I am not confident in the in vivo methylation data presented in Figure 1. As argued by the authors, this dataset should not be viewed as a resource for genome-wide changes in DNA methylation during neurogenesis but this is how the future readers will see it and use it. My suggestion is that it should be removed from the manuscript.

The dataset originally presented in Figure 1 has been removed from the revised manuscript together with all conclusions that were based on these data.

Point 2:
About the second part of the letter on maintenance methylation, I agree
with the author's response. OK for replacing "maintenance" by "pre-existing", but I would not include Figure R1 in the manuscript because this represents only 1.44% of CpGs (possible technical artefacts). In fact, it rather shows that 98,56% of CpGs do not show a pattern compatible with a maintenance function, indicating that Dnmt3a/b function is mainly related to de novo methylation.

As suggested we replaced the word „maintenance“ with „pre-existing“ in the text and figure legends referring to Fig. EV2C. We have added a sentence discussing possible sources of pre-existing methylation changes on page 9, lines 191-194, which reads as follows:

„Preexisting hypomethylation in KO NPCs could be explained by a differential adaptation of WT and KO NSPCs to the cell culture conditions or by a potential contribution of Dnmt3a/b to maintenance methylation during proliferation in vitro, i. e. by compensating propagation errors of Dnmt1 similarly as reported during passaging of ESCs (Liao et al, 2015)“.

The additional analysis looking at a maintenance role of Dnmt3a/b during differentiation has not been included in the revised manuscript, as suggested by referee and editor.

Additional changes to the manuscript:

Because the RRBS dataset of *in vivo* NSPCs, lPCs and neurons was removed from the manuscript, the following additional changes had to be introduced:

- Figure 1 was removed and replaced by an analysis of *in vitro* neurogenesis; main text, figure legend and Dataset EV1 were updated.
- Figure 3E was replaced with a panel showing the *in vitro* de novo CpGs; main text and figure legend were adjusted.
- Figure 4J was removed.
- The discussion section was updated and all comments referring to the *in vivo* dataset were removed.
- The abstract has been updated and now refers to the *in vitro* RRBS data instead of the *in vivo* data.
- Figures EV1, EV4, S1 and S3 were removed from the manuscript.
- Appendix Figure S2G is now presented in Appendix Figure S1.
- Figure 1H is now presented in Figure EV1.
- The methods section has been updated and all methods refering to the *in vivo* RRBS data set have been removed.
- Expanded View Datasets 1 and 2 were updated and Dataset EV3 was removed.
- Figure legends were modified according to the publisher’s suggestions.

Response to editorial requests:

- "Declaration of Interests" should be referred to as Conflict of Interest

This point has been changed.

- The author contributions are missing for Christine Steinhauer

The name has been corrected.

- Figure callouts are missing for Fig 7G, Fig EV3G & Fig EV5.

All missing callouts were added (Figure EV5 is Figure EV4 in the revised manuscript).

EMBOJ-2020-107100R

- We include a synopsis of the paper (see http://emboj.embopress.org/). Please provide me with a general summary statement and 3-5 bullet points that capture the key findings of the paper.

The summary statement and bullit points are now provided as a separate Word file.

- We also need a summary figure for the synopsis. The size should be 550 wide by [200-400] high (pixels). You can also use something from the figures if that is easier.

A summary figure has been uploaded.

2nd Revision - Editorial Decision 2nd Jul 2021

Dear Sara,

Thank you for submitting your revised manuscript to The EMBO Journal. I have now had a chance to take a careful look at everything and all looks good.

I am therefore please to accept the MS for publication here.

Congratulations on a nice study

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Please note that it is EMBO Journal policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper. If you do NOT want this, you will need to inform the Editorial Office via email immediately. More information is available here: https://emboj.embopress.org/about#Transparent_Process

Your manuscript will be processed for publication in the journal by EMBO Press. Manuscripts in the PDF and electronic editions of The EMBO Journal will be copy edited, and you will be provided with page proofs prior to publication. Please note that supplementary information is not included in the proofs.

Should you be planning a Press Release on your article, please get in contact with embojournal@wiley.com as early as possible, in order to coordinate publication and release dates.

If you have any questions, please do not hesitate to call or email the Editorial Office. Thank you for your contribution to The EMBO Journal.

** Click here to be directed to your login page: https://emboj.msubmit.net

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Sara Zocher
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2020-107100

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be iustified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumo

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Cellular analysis (cell culture and histology) were performed at least in triplicates with detailed sample sizes being specified in the Figure legends and in Appendix Table S5. Sample sizes were determined based on previous experience (cell culture, histology, sequencing) and by performing power calculations with the software G power based on previously determined effect sizes.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	See 1.a.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples were excluded after analysis. Dead or sick mice and histological stanings with insuffient quality were excluded from analysis.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Samples were randomized for all experiments. Mice of both genotypes received tamoxifen.
For animal studies, include a statement about randomization even if no randomization was used.	Samples were randomized for all experiments.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	All experiments were performed with the experimenter being blinded to the experimental groups.
4.b. For animal studies, include a statement about blinding even if no blinding was done	All behavioral test or animal treatmants were performed with the experimenter being blinded to the experimental groups.
5. For every figure, are statistical tests justified as appropriate?	Statistical tests were performed as appropriate and were described in the figure legends and methods section.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Normality was tested using Shapiro Wilk test based on data generated in the manuscript or based on count data from previous experiments. Additional results of non-parametric tests were included in Appendix Table S5.

Is there an estimate of variation within each group of data?	Standard errors of the mean are presented in every graph.
Is the variance similar between the groups that are being statistically compared?	Standard errors of the mean are presented in every graph. Variance was assessed using Graphpad Prism.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	All used antibodies were validated by the commercial providers. Please see the Appendix Table S3-4 for catalog numbers, providers and concentrations.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Cell lines were generated in the present study. Mycoplasma contamination was assessed in the cell culture suite on a regular basis.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Details are reported in the methods section of the manuscript.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All experiments were conducted in accordance with the applicable European and National regulations (Tierschutzgesetz) and were approved by the local authority (Landesdirektion Sachsen: file numbers 25-5131/354/63 and 25-5131/365/9).
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Confirmed.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Not applicable
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not applicable
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	Not applicable
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	Not applicable
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	Not applicable
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not applicable
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not applicable

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	RRBS and RNA sequencing data are deposited at GEO (GSE167955) as stated in the "Data Availability" section of the manuscript.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	Processed data tables are presented in Tables EV1-3.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	See 18.
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	Not applicable

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	Not applicable