EMBO reports

Paternal age affects offspring via an epigenetic mechanism involving REST/NRSF

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Dear Noriko,

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by two referees and their comments are provided below.

As you can see the referees find the analysis potentially interesting, but they also find that the work needs to be significantly extended in order to consider publication here. They appreciate the finding that REST genes seem to be involved in the described process, but we also get limited further insight into which genes in particular and the causality of this link for the phenotypes observed. Addressing this issue is not an easy task, but such analysis is needed to consider publication here. Given that it is unclear if the raised issues can be resolved, I am afraid that I see no other choice but to reject the present submission.

Should further work allow you to extend the findings along the lines as indicated by the referees then I am open to consider a new submission on this topic. I should point out that for re-submissions that we consider novelty at time of submission and might involve new referee(s) if needed. If you are interested in a resubmission please contact me beforehand to discuss the extent of the revisions.

For the present submission, I am sorry that I can't be more positive on this occasion.

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Referee #1:

Yoshizaki et al. investigate the impact of advanced parental age on the developing brain in the offspring. They compare offspring from 3 (YFO) and aged 12 month old fathers (AFO) and observe that AFO exhibit reduced ultrasound vocalization and mild alterations of cortical development at PND6. At the same time the analysis of sperm DNA from YF and AF revealed alterations in DNA-methylation, representing mainly hypo-methylation. The affected genomic regions are enriched for REST binding motifs. Gene-expression analysis of the developing forebrain (E14.5) reveals only very subtle changes in gene-expression, but GSEA hints towards the deregulation of REST target genes and other neuronal development pathways. Finally the authors treat young fathers with 5-AZA to mimic hypo-methylation and observe that the corresponding offspring exhibit ultrasound vocalization impairments.

The study addresses a very interesting topic. The finding that sperm DNA-methylation is affected by paternal age is not new, so the novelty of this study stems from the analysis of

the related mechanisms. The data appears sometimes somewhat preliminary and a number of questions should be addressed before the study can be published in EMBO J.

- Fig 1. The authors perform a number of tests on offspring from young fathers (YF0) and aged fathers (AFO). However, rather than reporting the "n" of the offspring used and litter sized greater than 6, they should consider the fact that phenotypes may vary due to the litters/mother. With other words, the fact that offspring come from different litters has to be considered in the statistical analysis.

- Fig. 2. Its not clear when the analysis was performed. It says " ...at the stage when impairment in vocal communication was observed...". Was it PND6?

- Fig. 3. The message to be communicate by the figure is at present not clear and appears to be out of context. How would the analysis of the 3 brain regions link to the defects seen in cortical development and cortical thickness? Is this also PND6? Moreover the panels are really misleading. It takes a while to understand that the 3 brain images shown in a-c should most likely simply reflect the brain regions analyzed. However, the images show an adult brain and analysis was done at PND6? Its furthermore unclear what panel d-e should show. What does Imi stand for and are we looking at the data from YFO or AFO? Same is true for panels g-i. I guess that panels J-o represent cFOS positive cells at baseline?

- Are the phenotypes described in Fig 1-3 affected by the gender of the offspring.

- The changes observed in sperm DNA-methylation are interesting due to the specificity of the effect linking to the REST target genes. The effects are comparatively mild. I suggest the authors have a look at previous study looking into sperm DNA from aged vs. young fathers (although father were older in these studies) also reporting reduced DNA-methylation (e. g. PMID 29467291).

- Selected DNA-methylation changes should be confirmed via other techniques, e.g. qPCR based methods.

- Fig. 4: As for the DMRs found in intergenic regions. How where they linked to corresponding genes.

- Fig. 5 I could not find information about the number of mice used for the RNA-seq. Also in the methods it states that embryonic telencephalons were isolated at E11.5 and E14.5. In the these it says that data from E14.5 is shown. Please clarify.

- Fig.5. The changes observed in gene-expression are very mild. At present its unclear how the gene-expression data is linked to the sperm DNA-methylation. The authors try to confirm DNA hypo-methylation in sperm and increased gene-expression in the E14.5 forebrain for genes detected by DNA-methylation analysis and present the data as EV7. Its not clear what is plotted in EV7. Is this qPCR data as stated in the text but not in the figure legends or methods? Why are there in contrast to EV4 only 6 genes linked to REST?

- Another possibility is that the data is diluted due to the fact that whole embryo forebrain was used, whereas the data suggest that specifically proliferating neuroprogenitor cells may be affected. Can the authors sort for such cells?

- Fig 6: This is an interesting experiment. To strengthen the link between global changes induced by 5-AZA and the rather specific alterations observed in sperm from old fathers it would be helpful to see which genomic regions are affected by 5-AZA treatment.

- Fig.6: What is the impact of 5.AZA on the developing brain (see Fig 2)?

- Can the authors discuss why specifically REST target genes would be affected by hypo-methylation in sperm?

Referee #2:

How the parental aging affects the behavior in next generation and the association with psychiatric diseases has been still largely unclear. In this paper, the authors presented that paternal aging in mice caused altered USV both in quantity and quality and it seemed to be one of the robust behavioral phenotypes related with psychiatric disorders caused by parental aging. The authors also found the DNA hypomethylation in sperm from aged male mice and the induction of DNA demethylation in young males also recapitulated the altered USV in the offspring. These findings may give a clue of the molecular mechanism that explains the risk of paternal aging in developmental disorders such as autism.

The authors found the thinner cortex of AFO, which recapitulates the observations in human ASD cases. This fact itself is very interesting and may be worth further investigating. However, it was not convincing to discuss the relationship with the altered USV in parallel. Fig. 2 suggested the cortical abnormality and Fig. 3 suggested the other brain regions as potential target brain regions. These different brain regions in the same study may become confusing. Although the reviewer agrees that c-fos phenotype is related to altered USV, discussing the abnormal brain structure and the neural activity in the other brain regions in parallel is difficult to follow the story.

A logic or the flow of this study is not smooth in this manuscript. As described in the above comment, Fig. 2 is split into the story of USV (Fig. 1 and 3). Gene set enrichment analyses in Fig. 5d-f are also related to the cortex story. It might be better to change the construction of figures. The order of figures will be, for example, Fig. 1, 3, 4, and 5a-c, gh, these are related to a story of USV and REST, and after Fig. 5d-f and before the final in vivo experiment (Fig. 6), Fig. 2 (maybe as a supplemental figure) can be inserted.

Did the authors confirm c-fos expression and the brain morphology in the pups born from 5-Aza treated young father? Because the authors suggested the difference of neural activity and brain morphology underlying the altered USV, they should be also addressed in this experiment. Ideally REST manipulation should be performed instead of 5-Aza treatment but it might be technically difficult. At least the authors should check the phenotypes of pups born from 5-Aza male animals besides USV.

Dear Editor.

Thank you for handling our previous manuscript entitled “Paternal age affects offspring’s traits via an epigenetic mechanism involving REST” submitted to *EMBO J*.

Although your editorial decision was “Reject”, we have substantially revised our manuscript according to reviewer’s valuable comments as below:

Based on the suggestion from the both reviewers, we have deleted original Figure 3, since neuronal activity was meaningful yet independent of the defects seen in the cortex in AFO. The figures were accordingly renumbered. We followed the Reviewer #2's suggestion and changed the order of Figure 2-4 in the current version. To respond his/her another suggestion, USV data of individual offspring were presented for each mother mouse in Extended View Fig.1. The revision has added solid evidence of the litter size as a confounding factor for USV phenotypes; the numbers of USV were lower when the litter size was less than 6. We deleted the original Extended View Fig.7 because this qPCR analysis targeting 94 genes chosen from hypo-DMRs was intended to determine the developmental stage for RNA-seq. The original Extended View Fig.8 was drastically rearranged to show results of RNA-seq analyses of the telencephalon at E11.5 in more detail (current Extended View Fig.7) to compare with those at E14.5 (current Fig.3).

We additionally performed bisulfite sequencing analyses of sperm DNA from the mice treated with de-methylation drug, 5-Azacitidine, and confirmed that 5-Azacitidine could in part (as in the case of *Shank2* and *Nav1*, well-known ASD genes) reproduce similar DNA hypo-methylation due to paternal aging (current Extended View Figures 9 and 10, Table S4, 5). We also changed the bar chart to the box plot on testis weight and immunofluorescence of 5-methylcytosine (current Figs.5b and d). Relatedly, we have added results of the immunohistochemical staining for 5-hydroxymethycytosine in the testis to further support the de-methylating effects of 5-Aza treatment (current Extended View Fig.8). We believe that these new data have strengthened our story on transgenerational epigenetic mechanisms of paternal aging.

We also discussed several points in response to reviewer’s comments. We followed the Reviewer #1's suggestion and discussed about a paper suggested by him/her [Xie et al., 2018]. Although aged sperm DNA also showed hypo-methylation, we did not find any genes in common in the vicinity of our hypo-DMRs. We suspect methodological difference as one possible explanation; we used SureSelect Methyl-Seq in our study, while they used a reduced representation bisulfite sequencing (RRBS) in the previous paper. Most importantly, our comprehensive whole genome data exhibited a specific enrichment of REST/NRSF binding motifs within hypo-DMRs in aged mouse sperm genome, which has not been found in any previous studies.

We followed the Reviewer #1's suggestion, and added discussion on hypo-DMRs and REST/NRSF binding motifs in aged mouse sperm. There are a few possibilities; because DNA methyltransferase 3b promotes REST/NRSF occupancy in developing heart [Zhang et al., 2017], DNA hypo-methylation may inhibit REST/NRSF binding. Alternatively, REST/NRSF binding may facilitate DNA hypo-methylation, as reported in embryonic stem cells [Stadler et al., 2011]. Relatedly, we added Extended View Fig.10 showing that *REST* mRNA is predominantly expressed in testicular stem cells (i.e., type A spermatogonia) based on a reanalysis of previous microarray data [Namekawa et al., 2006]. Although we could not uncover the molecular mechanism how DNA hypo-methylation occurs within REST/NRSF binding motives, our findings include biological significance, since it is not occurred randomly.

We followed the Reviewer #1's suggestion, discussed how DNA hypo-methylation affects gene expression, because most of the DMRs were located in intergenic regions. As supportive evidence, recently published interactome studies have revealed variants in noncoding regions that may impact on induction of phenotypes through perturbations in promoters and enhancers [Gallagher et al., 2018; Nott et al., 2019]. Likewise, functional *cis*-elements located hundreds of kilobases away from the coding regions can affect gene expression [Bulger and Groudine, 2011; Sanyal et al., 2012; West and Fraser, 2005]. Therefore, we can assume that the DMRs located in the intergenic regions may impact on gene expression. Accordingly, the references were numbered

As a result, we were able to reconstruct our manuscript to be straightforward by focusing on the molecular mechanisms involving REST/NRSF among various phenotypes due to paternal aging. The constructive comments by the reviewers allowed us to identify significant points in our manuscript that needed modification and clarification. Therefore, we consider that our revised manuscript has become much improved and is suitable for publication in *EMBO J.*

The revised text is indicated in red. Attached, please also find point-by-point responses to the reviewer's comments.

Due to the transfer of some of the authors have to another positions and contributions of additional experiments, the order of the authors has been changed.

We look forward to your editorial consideration.

Referee #1:

Yoshizaki et al. investigate the impact of advanced parental age on the developing brain in the offspring. They compare offspring from 3 (YFO) and aged 12 month old fathers (AFO) and observe that AFO exhibit reduced ultrasound vocalization and mild alterations of cortical development at PND6. At the same time the analysis of sperm DNA from YF and AF revealed alterations in DNA-methylation, representing mainly hypo-methylation. The affected genomic regions are enriched for REST binding motifs. Gene-expression analysis of the developing forebrain (E14.5) reveals only very subtle changes in gene-expression, but GSEA hints towards the deregulation of REST target genes and other neuronal development pathways. Finally the authors treat young fathers with 5-AZA to mimic hypo-methylation and observe that the corresponding offspring exhibit ultrasound vocalization impairments.

The study addresses a very interesting topic. The finding that sperm DNA-methylation is affected by paternal age is not new, so the novelty of this study stems from the analysis of the related mechanisms. The data appears sometimes somewhat preliminary and a number of questions should be addressed before the study can be published in EMBO J.

We appreciate the overall positive attitude of this reviewer for our manuscript.

- Fig 1. The authors perform a number of tests on offspring from young fathers (YF0) and aged fathers (AFO). However, rather than reporting the "n" of the offspring used and litter sized greater than 6, they should consider the fact that phenotypes may vary due to the litters/mother. With other words, the fact that offspring come from different litters has to be considered in the statistical analysis.

Thank you for your constructive comment. In the current version, we showed in Extended View Fig. 1 individual USV data for each mother. As you suggested, USV phenotypes varied among litters/mothers, suggesting a possible presence of confounding factors, and we used virgin female mice for mating with young or aged male mice to avoid possibilities that parenting experience affects USV calls in their pups. In our preliminary study, we noticed the number of littermates affected the USV phenotypes; the number of USV was decreased when the litter size was less than 6 as shown in Extended View Fig.1. That is why we only used data obtained from the offspring with the litter size of 6 or more. Therefore, we clarified the corresponding explanation in Results (p.5, line 86-97) and Figure legend (p.35, line 896-907).

- Fig. 2. Its not clear when the analysis was performed. It says " ...at the stage when impairment in vocal communication was observed...". Was it PND6?

Analyses of the brain structure (current Fig. 4) was performed at postnatal day 6 (PND6) when we measured USV. The information was added in the results (p.9, line 218-220).

- Fig. 3. The message to be communicate by the figure is at present not clear and appears to be out of context. How would the analysis of the 3 brain regions link to the defects seen in cortical development and cortical thickness? Is this also PND6? Moreover the panels are really misleading. It takes a while to understand that the 3 brain images shown in a-c should most likely simply reflect the brain regions analyzed. However, the images show an adult brain and analysis was done at PND6? Its furthermore unclear what panel d-e should show. What does Imi stand for and are we looking at the data from YFO or AFO? Same is true for panels g-i. I guess that panels J-o represent cFOS positive cells at baseline?

We are sorry for the confusion in the previous manuscript. We omitted the c-Fos data (previous Fig.3) in anxiety-related brain regions (i.e., the paraventricular thalamus (PVT), basolateral amygdala (BLA) and piriform cortex (Pir) in pups (PND6) [Kurumaji et al., 2003]), which was independent with the defects seen in cortical development in AFO. Relatedly, we reassigned new figure numbers (Fig.2-5). As a result, our revised manuscript became more logical and easier to understand. For your reference, the previous Figs. 3a-c show the three brain regions, and Figs. 3d-f show the number of c-Fos positive cells (i.e., activated neurons) immediately (Imi) and 2 hours after USV recording in the YFO. Figs. 3g-h show c-Fos positive cells in the PVT are correlated with USV. Figs. 3j-l show that the number of c-Fos positive cells was significantly decreased in PVT and BLA than those of AFO.

- Are the phenotypes described in Fig 1-3 affected by the gender of the offspring.

We totally agree that gender difference is one of the most important issues especially in ASD research. However, we did not observe significant gender difference in USV phenotypes in our preliminary experiments, and analyzed only male offspring here. We consider that this issue is the next most important challenge. Thank you for your suggestion.

- The changes observed in sperm DNA-methylation are interesting due to the specificity of the effect linking to the REST target genes. The effects are comparatively mild. I suggest the authors have a look at previous study looking into sperm DNA from aged vs. young fathers (although father were older in these studies) also reporting reduced DNA-methylation (e. g. PMID 29467291).

Thank you for your constructive comment and suggestion for the reference [PMID 29467291, Xie *et al.*, PNAS, 2018]. Consistent with our results and those in a previous literature [Milekic *et al.*, 2015], they found high levels of DNA hypo-methylation (299/484, ~62%) in promoter regions of the sperm DNA from old male mice. They showed a list of genes with hypo-methylated promoters (in their Table S1). Unfortunately, we could not find common genes with our gene sets near the hypo-DMRs. A reasonable explanation is a methodological difference; while they used a

conventional method, i.e., reduced representation bisulfite sequencing (RRBS), we applied a more modern technique, i.e., SureSelect Methyl-Seq, Agilent Technology covering a wider range of genome sequences than RRBS [Koike *et al.*, 2016; Miura *et al.*, 2015]. Therefore, we added detailed explanation by referring Xie et al. (2018), (p.11, line 281-296, reference #46). As a result, we were able to discuss in more depth how DNA hypo-methylation of sperm due to paternal aging affects gene expression in the next generation.

- Selected DNA-methylation changes should be confirmed via other techniques, e.g. qPCR based methods.

Thank you for your comments. In this study, we have chosen a DNA methylome analysis method (SureSelect Methyl-Seq) to minimize the amplification bias by PCR cycle. In addition, multiple samples were analyzed for each group to ensure reproducibility of the results. Given these facts, we do not consider verification by qPCR, which is prone to amplification bias, to be reasonable as a follow-up to the present analysis. Your suggestion encouraged us to notice that our methods are more appropriate. Relatedly, we have cited reference to motif analysis in Results (p.6, line 136-139, reference #24).

- Fig. 4: As for the DMRs found in intergenic regions. How where they linked to corresponding genes.

As you pointed out, most of the DMRs are in intergenic regions. Actually, it is difficult to examine impact of DMRs on expression of corresponding genes without deletion or modification experiments. However, several supporting papers have been reported. One is recently published interactome research, in which GWAS-identified risk variants in noncoding regions of the genome could exert phenotypic effects through perturbation of transcriptional gene promoters and enhancers [Gallagher et al., 2018; Nott et al., 2020]. Another evidence is that, many functional *cis*-elements are located hundreds of kilobases away from the coding region of target genes [Bulger and Groudine, 2011, Sanyal et al., 2012, West and Fraser, 2005]. In addition, our GSEA also revealed that gene sets near the DNA hypo-methylated regions (DMRs) were enriched in up-regulated genes in AFO (current Fig.3i, j). Therefore, we favor to imagine that DMRs could link to the nearest corresponding genes. This important discussion was added in the main text (p.11, line 310-p.12, line 318, reference #52-56).

- Fig. 5 I could not find information about the number of mice used for the RNA-seq. Also in the methods it states that embryonic telencephalons were isolated at E11.5 and E14.5. In the these it says that data from E14.5 is shown. Please clarify.

We conducted RNA-seq analyses using three telencephalic samples at both E11.5 and E14.5, each of which from different mothers. In contrast to data at E14.5, we did not detect enrichment of “late fetal genes”, “autism-related genes” etc. in E11.5 samples. In

the current version, we showed E11.5 and 14.5 data as Extended View Fig.7 and Fig.3, respectively. The corresponding explanation was added in Results and Legends for Ex View 7 (p.8, line 192-206, and p.8, line 212- p.9, line 215). In addition, we also described the results of E14.5 in detail (p.7, line 179-181). Thanks to this comment, we believe that our current version of the manuscript showing additional RNA-seq analyses data of E11.5 samples has become much improved.

- Fig.5. The changes observed in gene-expression are very mild. At present its unclear how the gene-expression data is linked to the sperm DNA-methylation. The authors try to confirm DNA hypo-methylation in sperm and increased gene-expression in the E14.5 forebrain for genes detected by DNA-methylation analysis and present the data as EV7. Its not clear what is plotted in EV7. Is this qPCR data as stated in the text but not in the figure legends or methods? Why are there in contrast to EV4 only 6 genes linked to REST?

We apologize that EV7 in the previous manuscript was misleading. The qPCR analyses using 94 genes related with DNA hypo-methylated regions (i.e., hypo-DMRs) in sperm was performed to determine the suitable embryonic stage for RNA-seq analyses. Since we found a clear tendency of leaky expression in E14.5 telencephalon, we actually performed RNA-seq at this stage. Because the data was less important, we omitted them data from the current version. As a result, our revised manuscript became more logical and easier to understand.

- Another possibility is that the data is diluted due to the fact that whole embryo forebrain was used, whereas the data suggest that specifically proliferating neuroprogenitor cells may be affected. Can the authors sort for such cells?

As you have wisely surmised, REST/NRSF specifically expressed in Sox2+ neural stem cells (NSCs) [Nechiporuk et al., 2016], and thus it would be ideal if we could enrich NSCs for RNA-seq analyses. However, a similar analysis has been performed by other group in the case of an autism model using the heterozygous *Chd8* knockout mice [Katayama et al., 2016]. From the same GSEA, they observed that early-fetal genes were enriched and abnormal activation of REST target genes. Therefore, our strategy is acceptable for comprehensive gene expression analyses.

- Fig 6: This is an interesting experiment. To strengthen the link between global changes induced by 5-AZA and the rather specific alterations observed in sperm from old fathers it would be helpful to see which genomic regions are affected by 5-AZA treatment.

Thank you for your constructive comment. We have added results showing global DNA hypo-methylation by 5-hydroxymethylcytosine staining of the testis (p.9, line 242-243, p.37, line 973-978, Fig.EV8). Relatedly, we reassigned new figure numbers (Fig.EV9-13). Additionally, we conducted DNA methylation analyses of sperm obtained from male mice treated with a de-methylation drug, 5-Aza. Eleven DMRs were selected

from 96 DMRs based on possible REST/NRSF binding sites by our *in sillico* ChIP analysis as well as differential gene expression by RNA-seq analyses. We revealed that the most of the target regions (8 in 11 targets) seemed to be hypo-methylated in sperm DNA from 5-Aza-treated male mice, in which a significant reduction of DNA methylation was detected at a CpG site of M2_hypo_003 (i.e., corresponding to *Nav1*) and M2_hypo_046 (corresponding to *Shank2*). Indeed, both *Nav1* (*Scn2a*) and *Shank2* are autism-related genes. We added the Extended View 9 and 10 and its corresponding explanation in Abstract (p.3, line 42-45), Introduction (p.4, line 77-80), Results (p.9, line 245-p.10, line 256), Materials and Methods (p.21, line 590-p.22, line 610, reference #75, 76), and Figure legend (p.37, line 980- p.38, line 1002). We have changed the literature numbers, since we referred methodological papers. Thanks to this comment, we were able to confirm that DNA hypo-methylation due to paternal aging could be reproduced by 5-Aza administrations.

- *Fig.6: What is the impact of 5.AZA on the developing brain (see Fig 2)?*

Thank you for your comments. This issue was beyond the scope of the current study and considered as the next challenge. Therefore, we did not mention the brain development in pups derived from father with 5-Aza injections.

- *Can the authors discuss why specifically REST target genes would be affected by hypo-methylation in sperm?*

We appreciated the comment from this reviewer. At this moment, we cannot provide actual data to answer his/her question. However, here are several references that we think informative. By analyzing a previous study [Namekawa et al., 2006], we found that *Rest* mRNA is actually expressed in male germline cells and the expression level was highest in testicular stem cells (i.e., type A spermatogonia) as shown in Extended View Fig.10. Recent single-cell RNA sequencing data from testicular cells have also confirmed a distinct high expression of *Rest* in the most immature sub-population of spermatogonia [Green et al., 2018]. It is reported in embryonic stem cells that REST/NRSF binding induces DNA hypo-methylation in the neighboring genome region [Stadler et al., Nature, 2011]. In the developing heart, DNA methyltransferase 3b promotes REST/NRSF occupancy [Zhang et al., 2017]. Although our current study could not reveal how DNA hypo-methylation occurs within REST/NRSF binding motives, our findings clearly demonstrated that DNA hypo-methylation in sperm from aged mice does not occur randomly, but is biologically significant. It will be the next challenge how REST/NRSF molecules are recruited within DNA hypo-methylated regions. The point suggested by this reviewer has provided us with new knowledge and we could discuss in more depth. We added this important discussion in Discussion (p.11, line 297-309, reference #48-51) and Figure legend (p.38, line 1004-1007).

Referee #2:

How the parental aging affects the behavior in next generation and the association with psychiatric diseases has been still largely unclear. In this paper, the authors presented that paternal aging in mice caused altered USV both in quantity and quality and it seemed to be one of the robust behavioral phenotypes related with psychiatric disorders caused by parental aging. The authors also found the DNA hypomethylation in sperm from aged male mice and the induction of DNA demethylation in young males also recapitulated the altered USV in the offspring. These findings may give a clue of the molecular mechanism that explains the risk of paternal aging in developmental disorders such as autism.

The authors found the thinner cortex of AFO, which recapitulates the observations in human ASD cases. This fact itself is very interesting and may be worth further investigating. However, it was not convincing to discuss the relationship with the altered USV in parallel. Fig. 2 suggested the cortical abnormality and Fig. 3 suggested the other brain regions as potential target brain regions. These different brain regions in the same study may become confusing. Although the reviewer agrees that c-fos phenotype is related to altered USV, discussing the abnormal brain structure and the neural activity in the other brain regions in parallel is difficult to follow the story.

This has also been pointed out by Referee #1. The brain regions focused in the previous manuscript (i.e., the paraventricular thalamus (PVT), basolateral amygdala (BLA) and piriform cortex (Pir)) are related with anxiety in pups [Kurumaji et al., 2003], and different from the cortex, in which we revealed abnormal structures. To avoid confusion, we omitted the data in the present manuscript in the current manuscript. Relatedly, we reassigned new figure numbers (Fig.2-5). As a result, our revised manuscript became more logical and easier to understand.

A logic or the flow of this study is not smooth in this manuscript. As described in the above comment, Fig. 2 is split into the story of USV (Fig. 1 and 3). Gene set enrichment analyses in Fig. 5d-f are also related to the cortex story. It might be better to change the construction of figures. The order of figures will be, for example, Fig. 1, 3, 4, and 5a-c, g, h, these are related to a story of USV and REST, and after Fig. 5d-f and before the final in vivo experiment (Fig. 6), Fig. 2 (maybe as a supplemental figure) can be inserted.

Thank you for your constructive comments. Since abnormal and ectopic expressions of REST/NRSF target genes and late-fetal gene implicate impaired development of the brain, your suggestion is logical and easy to understand. Therefore, we revised the order of figures and the corresponding text between results of DNA methylome and RNA-seq analyses (p.7, line 160-167). Relatedly, we reassigned new figure numbers (Fig.2-5). In relation, we added the proportion of cortical hypoplasia and statistical values (p.9, line 221-227), and added references that show a link between smaller brain and impairment

in REST pathways (p.9, line 229-230, reference #40, 41). Accordingly, we revised Figure numbers and legend (p.33, line 853-p.34, line 883) in manuscript.

Did the authors confirm c-fos expression and the brain morphology in the pups born from 5-Aza treated young father? Because the authors suggested the difference of neural activity and brain morphology underlying the altered USV, they should be also addressed in this experiment. Ideally REST manipulation should be performed instead of 5-Aza treatment but it might be technically difficult. At least the authors should check the phenotypes of pups born from 5-Aza male animals besides USV.

We agree with your suggestions. It would be ideal to analyze brain morphology and behaviors other than USV in the offspring derived from 5-aza treated mice. REST itself and its related molecules have already been proven its importance in brain development and neurodevelopmental diseases [Ballas et al., 2005; Lunyak et al., 2005]. However, it would technically difficult to manipulate REST in male germline cells and to observe its effect in the next generation. These issues were thus beyond the scope of the current study and considered as the next challenge.

Dear Noriko,

Thank you for submitting your revised manuscript to The EMBO Journal. I am sorry for the delay in getting back to you with a decision, but I have now received the two referees reports on the revised version and I am afraid that the overall opinion is not very positive.

Both the referees appreciate the added data, but referee #1 is also not convinced that the revisions go far enough to address the raised concerns. In particular, we still gain too limited mechanistic insight into how aging selectively effects DNA methylation within REST/NRSF binding motives. I know that addressing this issue is not straight forward and we wouldn't need the whole mechanism but some further understanding of this would be needed for consideration here.

I have discussed the manuscript further with my colleague Esther Schnapp at EMBO Reports and EMBO Reports is interested in the manuscript. They would involve one additional advisor to get input on the study and the conclusiveness of the 5-Aza experiment. If you are interested in this option please contact Esther eschnapp@wiley.com to discuss this further.

I am very sorry that I can't be more positive for The EMBO Journal, but I hope that you will consider the EMBO Reports option.

1st Editorial Decision 18th Aug 2020

Dear Dr. Osumi,

Thank you for the transfer of your research manuscript to EMBO reports. I now went through your manuscript, the referee reports from The EMBO Journal (attached again below), and your final revision plan. Both referees acknowledge that the findings are of interest. Nevertheless, mainly referee #1 has raised a number of concerns and suggestions to further improve the manuscript, or to strengthen the data and the conclusions drawn.

EMBO reports emphasizes novel functional over detailed mechanistic insight, but asks for strong in vivo relevance of the findings, and clear experimental support of the major conclusions. Thus, we will not require that points regarding more refined mechanistic details experimentally be addressed. In that light, it will be necessary that in final revised manuscript you address the points 1-3, 5 and 6 of referee #1 experimentally and/or by implementing the suggested changes (as indicated in your p-b-p-response).

I agree with referee #1 (his/her point 4), though, that the data showing that 5-AZA treatment phenocopies the age by derepressing/demethylating the RSET signature genes is weak. It seems there is only one CpG in Shank1 that is contained in the RSET binding sequence that shows clear demethylation (Fig. EV9). I would have expected a stronger and more general effect here, if it is the hypomethylation of these genes that causes the phenotype. Thus, the real causal targets that get demethylated by 5-AZA to copy the phenotype might be completely different. I understand that extensive further experimentation would be needed to address this in more detail. Thus, in case you do not have more convincing data, I would ask you to tone down very explicitly the conclusion that 5-AZA-treatment induces hypo-methylation of the same genes that show altered expression in the embryonic forebrain of the offspring of aged fathers (and remove that statement from the abstract). In that light, please also modify the discussion.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript and/or in a detailed point-by-point response. Acceptance of your manuscript will depend on a positive outcome of the next, final round of review.

Revised manuscripts should be submitted within three months of a request for revision. We are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic and we have therefore extended our 'scooping protection policy' to cover the period required for full revision. Please contact me to discuss the revision should you need additional time, and also if you see a paper with related content published elsewhere.

When submitting your revised manuscript, please also carefully review the instructions that follow below.

PLEASE NOTE THAT upon resubmission revised manuscripts are subjected to an initial quality control prior to exposition to re-review. Upon failure in the initial quality control, the manuscripts are sent back to the authors, which may lead to delays. Frequent reasons for such a failure are the lack of the data availability section (please see below) and the presence of statistics based on n=2 (the authors are then asked to present scatter plots or provide more data points).

When submitting your revised manuscript, we will require:

1) a .docx formatted version of the final manuscript text (including legends for main figures, EV figures and tables), but without the figures included. Please make sure that changes are highlighted to be clearly visible. Figure legends should be compiled at the end of the manuscript text.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure), of main figures and EV figures. Please upload these as separate, individual files upon re-submission. You can have up to 8 main figures.

The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf file labeled Appendix. The Appendix should have page numbers and needs to include a table of content on the first page (with page numbers) and legends for all content. Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures and tables according to this nomenclature.

For more details please refer to our guide to authors:
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See also our guide for figure preparation:
http://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14693178/authorguide). Please insert page numbers in the checklist to indicate where the requested information can be found in the manuscript. The completed author checklist will also be part of the RPF.

Please also follow our guidelines for the use of living organisms, and the respective reporting guidelines: http://www.embopress.org/page/journal/14693178/authorguide#livingorganisms

5) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq and array data) are deposited in an appropriate public database. This is now mandatory (like the COI statement). If no primary datasets have been deposited in any database, please state this in this section (e.g. 'No primary datasets have been generated and deposited').

See also: http://embor.embopress.org/authorguide#datadeposition

Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Methods) that follows the model below. Please note that the Data

Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

Moreover, I have these editorial requests:

6) We strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. If you want to provide source data, please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

7) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at: http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

8) Regarding data quantification and statistics, can you please specify, where applicable, the number "n" for how many independent experiments (biological replicates) were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. Please provide statistical testing where applicable, and also add a paragraph detailing this to the methods section. See: http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

9) Please note our new reference format: http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

10) Please have your final revised manuscript carefully proofread by a native speaker.

11) Please make sure that all microscopic images have scale bars that are defined in the respective figure legend.

12) Please reduce the number of key words on the title page to 5.

Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript. Please find instructions on how to link the ORCID ID to the account in our manuscript tracking system in our Author guidelines:
http://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines

In addition I would need from you:
- a short, two-sentence summary of the manuscript (less than 40 words)
- two to three bullet points highlighting the key findings of your study
- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Kind regards,

Achim

Achim Breiling
Editor
EMBO reports

Referee #1:

Yoshizaki et al. present this manuscript as a resubmission. The presented data is still very interesting and the authors tried to address all issues raised by the reviewers and performed a substantial amount of new experiments, which I really appreciate. However, I still have the feeling that the phenotypes and sperm DNA-methylation changes - which are not entirely novel - are not yet backed by the mechanistic studies, which would be the exciting novelty of this study. The key-message of this manuscript is in my view that being and old (mouse) father affects the expression of REST-linked gene-expression in the offspring embryonic brain in a DNA-methylation dependent manner which affect brain development at the structural level and that this can be phenocopied by systemic administration of a DNA-methylation inhibitor. Unfortunately, I think even the novel data do not really strengthen this interpretation. However, I appreciate the importance of transgenerational experiments and understand how time consuming these experiments are so I am happy to be convinced otherwise by my fellow reviewers.

Here are some still remaining questions:

1. A previous suggestion was, that the authors should take into account the litter size in the statistical analysis of the USV data. I appreciate that the author addressed this point and identify "litter size" as a confounding factor. As a consequence, the authors state that only data from mothers with a littersize n>6 was considered. I am not sure this would be a proper approach. As can be seen from novel FIG EV1 the variability in USV is even greater in offspring from young fathers when the littersize is n>6 when compared to n<6. Maybe my previous comment was not entirely clear. Rather than using an arbitrary cut off it would be more feasible to use all data but correct for litter size as a confounding factor in a statistical analysis. This approach is often used in

transgenerational studies and would help to gain confidence in the observed changes, that are - as in most transgenerational datasets - comparatively mild.

2. Fig 2B. I guess the heat map is based on hierarchical clustering. Please add the dendrogram to the panel.

3. I appreciate the data shown in what now became Fig 3. I am also willing to accept that the very mild changes (no significant changes are observed via diff expression analysis) observed could have relevant impact on the pathways presented. In the previous version of the manuscript the number of samples employed in the RNA-seq experiment was not presented. We now learn that n=3 was used for the experiments which I would consider "borderline" of what would now be acceptable for such type of experiments. I suggest that the authors present at least a PCA analysis and consider confounding factors such as batch effects etc. to strengthen their data and address these issues in more detail in the methods section.

4. I am glad to see that the authors tried to address the question if systemic administration of 5-Aza affect DNA-methylation in sperm. However, I do not think that the semi-quantitative analysis of 5mC via immunohistochemistry as shown in the main figure 5 is helpful in this regard. The authors performed targeted bisulfite sequencing for 11 genes linked to hypo-methylation and REST binding. In contrast to the text I do not see changes in any of the genes shown in Fig EV10 and only in 2 of the 11 genes, namely Nav1 and Shank there are significant changes, but only if the authors bin the genomic region a somewhat biased manner. At least in my view, the presented data does not provide strong evidence that 5-Aza acts via the mechanism described in Fig. 1-4.

5. The sentence "line 166-169 should be re-written. Its not clear at present that AFO was compared to YFO and that the message would be that there are NO significant changes.

6. Typo line76 "mail mice"

Referee #2:

First of all, there are mistakes and insufficient responses in a point-by-point response. For example, page and line numbers the authors described are not corresponded to the final text. In my last comment, I suggested showing other phenotypes of pups born from 5-Aza male animals besides USV. Actually the authors seemed to check DNA methylation in the selected hypo-DMRs by bisulfite sequencing and found significant reduction of the DNA methylation ratio in autism-related genes, but they did not mention it in the response. Overall, the authors basically responded to my comments and the revised version has improved.

Typo: in the of selected hypo-DMRs (p.8, line 243)

Referee #1:

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Here are some still remaining questions:

1. A previous suggestion was, that the authors should take into account the litter size in the statistical analysis of the USV data. I appreciate that the author addressed this point and identify "litter size" as a confounding factor. As a consequence, the authors state that only data from mothers with a littersize n>6 was considered. I am not sure this would be a proper approach. As can be seen from novel FIG EV1 the variability in USV is even greater in offspring from young fathers when the littersize is n>6 when compared to n<6. Maybe my previous comment was not entirely clear. Rather than using an arbitrary cut off it would be more feasible to use all data but correct for litter size as a confounding factor in a statistical analysis. This approach is often used in transgenerational studies and would help to gain confidence in the observed changes, that are - as in most transgenerational datasets - comparatively mild.

Thank you for your critical comments for improving our manuscript. We performed additional analysis of the rest of USV data from those with less than six littermates and combined all the data. Consistently, we could confirm that the

number of USV (Fig.1C), the number of syllable types (Fig.1I) and normalized entropy scores (Fig.1J) were decreased in aged father-derived offspring (AFO) compared with young father-derived offspring (YFO). Likewise, most of the syllable types were similarly changed; the percentage of “Downward” was increased, while those of “One jump”, “Chevron, “Upward”, “More jump”, “Wave”, and “More jump + Harmonics” syllables were decreased (Fig.1H). In this analysis, however, the average of body weight in AFO was not statistically different from that in YFO (Fig.1B). We accordingly revised our manuscript in Results (p.5, line 111-114, p.5, line 118-p.6, line 134) and Figure Legends (p.36, line 955-966).

2. Fig 2B. I guess the heat map is based on hierarchical clustering. Please add the dendrogram to the panel.

Genome regions shown as a heat map (Figure 2B) were not “clustered” but just aligned according to their chromosomal regions, and therefore, we cannot draw a dendrogram. To avoid misleading, we added the explanation in Results (p.6, line 147-149) and Figure Legends (p.36, line 979-980).

3. I appreciate the data shown in what now became Fig 3. I am also willing to accept that the very mild changes (no significant changes are observed via diff expression analysis) observed could have relevant impact on the pathways presented. In the previous version of the manuscript the number of samples employed in the RNA-seq experiment was not presented. We now learn that n=3 was used for the experiments which I would consider "borderline" of what would now be acceptable for such type of experiments. I suggest that the authors present at least a PCA analysis and consider confounding factors such as batch effects etc. to strengthen their data and address these issues in more detail in the methods section.

We appreciate that this reviewer is positive for our transcriptomic data. We paid close attention to collect embryos for RNA-seq at the same developmental stage based on the number of somites, even though they were isolated from different mothers (see Materials and Methods, p.20, line 570-572). Therefore, variations between samples causing “batch effects” are considered to be relatively small.

According to this reviewer's suggestion, we additionally performed principal component analysis (PCA), and found globally distinct expression profiles between YFO and AFO (see additional new Fig. EV2). We thus revised our manuscript describing this key finding in Results (p.8, line 196-198), Materials and Methods (p.20, line 591-592) and Figure Legends (p.40, line 1068-1075). We would like to thank the reviewer for his/her great insight.

4. I am glad to see that the authors tried to address the question if systemic administration of 5-Aza affect DNA-methylation in sperm. However, I do not think that the semi-quantitative analysis of 5mC via immunohistochemistry as shown in the main figure 5 is helpful in this regard. The authors performed targeted bisulfite sequencing for 11 genes linked to hypo-methylation and REST binding. In contrast to the text I do not see changes in any of the genes shown in Fig EV10 and only in 2 of the 11 genes, namely Nav1 and Shank there are significant changes, but only if the authors bin the genomic region a somewhat biased manner. At least in my view, the presented data does not provide strong evidence that 5-Aza acts via the mechanism described in Fig. 1-4.

We admit that this part is insufficient to draw a firm conclusion. We searched a comprehensive DNA methylome analysis dataset in sperm from mice treated with a de-methylation drug (5-Aza) and found a public dataset using reduced representation bisulfite sequencing (RRBS) (Kläver et al, 2015). Unfortunately, due to sparce signals in RRBS, only 21 DMRs were calculable among 96 hypo-DMRs that we identified by our SureSelect Methyl-Seq, Agilent Technology (Koike et al, 2016).

Interestingly, however, 12 of the DMRs, including those within *Nav1*, were significantly hypo-methylated in 5-Aza-treated sperm. From our analysis, we found that *Nav1* actually has a hypomethylated CpG site containing not the REST binding site itself but a binding motif of Specificity Protein1 that can interact with REST for regulating expression of its target genes (Paonessa et al, 2013; see revised Fig. EV4A). Relatedly, we noticed that *Shank2* contains CpG site neighboring the REST binding motif (Fig. EV4B).

Longitudinal cohort analyses in the US have previously suggested that methylation status of sperm DNA is associated with autism risk in offspring (Feinberg et al, *Int J Epidemiol,* 2015). A study has also observed that de-methylation of genome regions is transmitted from father to offspring (Atsem et al, *Human Mol Genet*, 2016). We consider that our paternal aging model is more natural than genetically engineered mice that have deficiency in certain genes. However, the most struggling issue is that we cannot precisely correlate a hypo-methylation at a certain genome region of sperm from aged father with its outcome at the level of the developmental program and subsequent behavioral phenotypes in the offspring. Since we cannot perform additional experiment at this time point, we would of course agree to tone down our interpretation.

We described above issues in Results (p.10, line 274-287) and Discussion (p.14, line 386-394). In relation, we have toned down our arguments in Abstract (p.3, line 51-56), Introduction (p.4, line 85-p.5, line 101) and Discussion (p.14, line 414-p.15, line 429)

5. The sentence "line 166-169 should be re-written. Its not clear at present that AFO was compared to YFO and that the message would be that there are NO significant changes.
We agree that expression changes of individual genes between YFO and AFO were very little. Therefore, we applied to GSEA, as in the case of a previous study on a genetically engineered autism model mice (Katayama et al, Nature, 2016). Fortunately, we were able to detect global changes in expression of gene sets, which actually resulted in identification of interesting phenotypes such as common genes with REST target genes, ASD related genes and genes related with precocious neurogenesis. We rewrote the manuscript in Results (p.8, line 193-196).

6. Typo line76 "mail mice"
We revised our typo (p.5, line 98).

Referee #2:

First of all, there are mistakes and insufficient responses in a point-by-point response. For example, page and line numbers the authors described are not corresponded to the final text. In my last comment, I suggested showing other phenotypes of pups born from 5-Aza male animals besides USV. Actually the authors seemed to check DNA methylation in the selected hypo-DMRs by bisulfite sequencing and found significant reduction of the DNA methylation ratio in autism-related genes, but they did not mention it in the response. Overall, the authors basically responded to my comments and the revised version has improved.

We apologize that the numbers for pages and lines were not corresponding to the final text, which may be due to the conversion when we uploaded the text file. Since two top authors have already left our laboratory, it is very difficult to perform additional experiments to observe other behavior phenotypes of the offspring derived from the male mice treated with a de-methylation drug. We did describe about our data on bisulfite sequencing data (in new Figs.EV4,5), in Results (p.10, line 274-287), and Figure Legend (p.40, line 1090-p.41, line 1122).

Typo: in the of selected hypo-DMRs (p.8, line 243)

We revised the typo as “in the selected hypo-DMRs” (p.10, line 274).

1st Revision - Editorial Decision 21st Oct 2020

Dear Prof. Osumi,

Thank you for the submission of your revised manuscript to our editorial offices. As the original referee #1 was unresponsive to my invitations to re-assess the study, we have discussed the revised manuscript and your point-by-point response in the team. We think that the remaining concerns of the referees have been adequately addressed, either by additional data or by text changes, and have thus decided to proceed with the manuscript.

Before formal acceptance, I ask you to address the following editorial requests in a final revised manuscript:

- I would suggest this shortened title:
Paternal age affects offspring via an epigenetic mechanism involving REST

- Please provide the abstract written in present tense.

- The key words are shown twice. Please remove these from the title page. It is fine to show the key words after the abstract. Please restrict these to 5.

- There are still several typos and grammatical errors in the text. Please have your final manuscript carefully proofread by a native speaker.

- Please make sure that the datasets indicated in the Data Availability Section (DAS) are indeed deposited and accessible. Please also add the URL for each dataset to the DAS.

- Regarding data quantification and statistics, please make sure that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends (also of the EV figures). Please also check that all the p-values are explained in the legend, and that these fit to those shown in the figure. Please provide statistical testing where applicable. For example, in Fig. EV5 it is completely unclear how many replicates are shown, and why no statistics is shown.

- The figure legends are often very short (see e.g. those for Fig. 1). Please add more details that also non-expert readers can follow. E.g. please explain better what is shown in 1B-1G.

- Please call out the single panels if Figs. EV2, EV4 and EV5 in the manuscript text.

- Please name the Tables S1-S5 'Dataset EV1 - Dataset EV5' and change their callouts in the manuscript text. Please also provide a legend for each dataset file and put this on the first TAB of the excel file (not in the main manuscript text).

- Please check that all microscopic images have scale bars and that their size is defined in the respective figure legend (e.g. Fig. EV3A has no scale bars).

- Could the images in Fig. 6A and 6C be shown in the same size?

- For those diagrams where statistical testing has been done, but the differences are not

significant, please indicate this using 'n.s.' as in Fig. EV3D/E.

- For Fig. EV3C you indicate that data from two replicates are shown. Thus, please do not combine the datasets, but show them as individual bars and remove the error bars.

- Finally, please find attached a word file of the manuscript text (provided by our publisher) with changes we ask you to include in your final manuscript text, and some queries, we ask you to address. Please provide your final manuscript file with track changes, in order that we can see any modifications done.

In addition, I would need from you:
- a short, two-sentence summary of the manuscript
- two to four bullet points highlighting the key findings of your study
- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript. Please do that for corresponding author Kono. Please find instructions on how to link the ORCID ID to the account in our manuscript tracking system in our Author guidelines:
http://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

Kind regards,

Achim

Achim Breiling
Editor
EMBO Reports

The authors have addressed all minor editorial requests.

2nd Revision - Editorial Decision 25th Nov 2020

Prof. Noriko Osumi
Tohoku University School of Medicine
Center for Neuroscience
2-1, Seiryo-machi
Aoba-ku
Sendai 980-8575
Japan

Dear Prof. Osumi,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

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If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: emboreports@embo.org]. If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

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

Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,

Achim Breiling
Editor
EMBO Reports

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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: Noriko Osumi
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2020-51524V2

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.

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?	We did not use statistical methods to estimate sample size.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	We described sample size in each experiment.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No. We did not exlude any data and used all data we got.
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.	No.
For animal studies, include a statement about randomization even if no randomization was used.	We did not use randomization, since we used all data.
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.	Yes. We conducted data acquisition and analysis by several researchers to eliminate subjective bias.
4.b. For animal studies, include a statement about blinding even if no blinding was done	We conducted data acquisition and analysis by several researchers and used "identification" to manage the data.
5. For every figure, are statistical tests justified as appropriate?	Yes. We used an appropriate statistical test for every figure.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We used different statistical methods according to normal distribution and homoscedasticity.
Is there an estimate of variation within each group of data?	No. We used all data we obtained.

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/

Is the variance similar between the groups that are being statistically compared?	Yes. There is some variance between the groups in our data, which may be attributed to differnece in the mean.

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).	We described antibody catalog number and references.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	We did not use cell lines in our study.

* 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.	We used C57BL/6J mouse with both gender for behavioral analysis, while we used male C57BL/6J mouse for DNA methylome in sperm, transcriptome in developing mouse and histological analysis in early postnatal. Mice were group housed. One male mouse were mated with one or two female mouse for 1 week. After then, male mouse was removed from breading cage.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All experimental procedures were approved by Ethic Committee for Animal Experiments of Tohoku University Graduate School of Medicine (#2014-112) as well as of Tokyo University of Agriculture, and animals were treated according to the National Institutes of Health guidance for the care and use of laboratory animals.
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.	We confirmed compliance.

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	DNA methylome data: DRA007933, RNA-sequence data: DRA008023
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).	We deposited a public database on DNA methylome data and RNA-sequence data as mentioned above.
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).	We did not use human clinical and genomic datasets.
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.	We did not use computational models.

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.	No.