EMBO reports

Glial Hedgehog signalling and lipid metabolism regulate neural stem cell proliferation in Drosophila

Qian Dong, Michael Zavortink, Francesca Froldi, Sofya Golenkina, Tammy Lam, and Louise Cheng
DOI: 10.15252/embr.20 2052130

Corresponding author(s): Louise Cheng (louise.cheng@petermac.org)

Review Timeline:

Transfer from Review Commons:	19th Nov 20
Editorial Decision:	20th Nov 20
Revision Received:	4th Jan 21
Editorial Decision:	10th Feb 21
Revision Received:	11th Feb 21
Accepted:	12th Feb 21

Editor: Martina Rembold

Transaction Report: This manuscript was transferred to EMBO reports following peer review at Review Commons.

(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 20th Nov 2020

Dear Louise,

Thank you for the submission of your research manuscript to EMBO reports. It has been reviewed by the journal-independent platform Review Commons and you have submitted a point-by-point response and outlined a revision plan.

We agree that your study is potentially a nice contribution to EMBO reports and we therefore invite you to revise your manuscript along the lines outlined in your revision plan. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

We invite you to submit your manuscript within three months of a request for revision. This would be February 20th, 2021 in your case. However, we are aware of the fact that many laboratories are not fully functional due to COVID-19 related shutdowns and we have therefore extended the revision time for all research manuscripts under our scooping protection to allow for the extra time required to address essential experimental issues. Please contact us in case you need more time so that we can discuss timing and revisions further.

IMPORTANT NOTE: we perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:

1) A data availability section is missing.
2) Your manuscript contains error bars based on n=2. Please use scatter blots showing the individual datapoints in these cases. The use of statistical tests needs to be justified.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

When submitting your revised manuscript, we will require:

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).
Please download our Figure Preparation Guidelines (figure preparation pdf) from our Author Guidelines pages
https://www.embopress.org/page/journal/14693178/authorguide for more info on how to prepare your figures.

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 (). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist

will also be part of the RPF.

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 your ORCID ID to your account in our manuscript tracking system in our Author guidelines
()

6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here:

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

7) Please note that a Data Availability section at the end of Materials and Methods is now mandatory. In case you have no data that requires deposition in a public database, please state so instead of refereeing to the database.
See also < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>).
Please note that the Data Availability Section is restricted to new primary data that are part of this study.

8) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available .

9) 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 .

10) Regarding data quantification
The following points must be specified in each figure legend:
- the name of the statistical test used to generate error bars and P values,
- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,
- the nature of the bars and error bars (s.d., s.e.m.)

Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.
- Please also include scale bars in all microscopy images.

11) As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

You are able to opt out of this by letting the editorial office know (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."

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

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,
Martina

Martina Rembold, PhD
Senior Editor
EMBO reports

We thank the reviewers for their useful suggestions, we have carried out a number of experiments and have amended the manuscript to clarify a number of these points.

Reviewer #1

Major comments:

1.Since Hh RNAi decreases the glial compartment (which slows NB proliferation) and increases the frequency of pH3+ NBs, it is unclear why it would decrease the number of EdU+ NBs (Fig. S3C).

Our experimental data suggests that accompanying glial niche disruption and downregulation of glia-derived signals, NBs are stalled in M phase (we detected an increase in the percentage of $pH3^+$ NBs). As a consequence, fewer NBs are in G1 and S phase. Therefore, we observed a reduction in EdU incorporation. This NB phenotype (increase in pH3 index and decrease in EdU index) was also observed by Speder and Brand, 2018, when they induced glial niche impairment by inhibiting the PI3K signaling pathway.

To address whether glial-Hh knockdown reduces the ability of NBs to produce progeny, we have carried out a new EdU pulse chase experiment (Figure EV3 H-K), where we assessed the number of progeny produced in a 3-hr time window (NB lineage marked by Pros-GFP) under control and glial-Hh knockdown conditions. We found a 25% reduction in EdU^+ cells per lineage, suggesting that glial niche impairment mediated by Hh knockdown reduces the speed by which NBs generate neurons.

2. If overexpression of htl[ACT] slows the NB cell cycle (as evidenced by reduced pH3 and EdU positive cells), it unclear why it does not reduce the number of NBs (Fig. 4L).

The number of NBs in the larval CNS is specified at the beginning of post-embryonic neurogenesis, when quiescent NBs re-enter the cell cycle (reviewed by Homem and Knoblich, 2012). Once NBs re-enter the cell cycle, the number of NBs remain constant. NBs undergo asymmetric division to produce one daughter NB and a GMC, which divides once to generate two neurons. Changes in the NB cell cycle speed does not alter the overall NB

number, only the number of neurons produced. To clarify this, we add a schematic depicting NB asymmetric division, Fig 1A.

3.What is the justification for presenting the EdU quantifications as an EdU index in which the experimental values are normalized to the average number of positive cells in the control?

EdU index is calculated as number of EdU^+ NBs normalised to control EdU^+ NBs. The number of EdU^+ NBs reflects the NBs that progress through S phase in a 15-min time window relative to control. A similar method was used in Kanai et al., 2018. This method would not be valid only if NB number varied between control and experimental data sets, however, the number of NBs in all our genetic manipulations are not significantly altered relative to their control (Reviewer_Fig 1A, B, the same as Fig EV3G and Fig 4L).

As regards to why we normalise to control in each of these experiments, this is because *in-vitro* EdU incorporation rely on Click-IT chemistry, which is inherently variable due to incubation conditions. To overcome this, we always incubate control and experimental brains in the same tube and imaged them with the same confocal setting, and each experiment is normalised to its control done in parallel. We have now included Reviewer's Fig Table 1 which includes average EdU^+ NB number, SEM and sample size from these experiments. The raw data is also included as source data with each main figure. We have also explained in more details in the Methods section regarding how the EdU experiments were carried out (Line 542-543).

4. In many cases, the comparison is to the same w [1118] line so it does not control for a specific genetic backgrounds and yet this method may be obscuring experimental variation present between datasets.
We have used three different controls in our experiments, 1) *GAL4* or *lexA* >w^{1118}, 2) *GAL4* or *lexA* > *mcherryRNAi*, 3) *GAL4* or *lexA* > *luc*. We have detected no significant difference in the raw EdU^+ NB counts between all the controls used in our experiments (Reviewer_Fig 1C) [Figures for referees not shown.]. In our revised manuscript, we have included in the Methods section a sentence clarifying this: "We have utilised GAL4 driver > w^{1118}, *UAS-mcherryRNAi* or *UAS-luc* interchangeably as controls in our experiments, as we found EdU incorporation did not significantly alter between these controls" (Line 630-633).

5. Likewise, why is glial number presented as a fold-change but NB number is presented as raw counts (e.g. 2D vs S3E)?

As the number of glial cells is far greater than what can be determined via manual counting, we utilised a Fiji 3D object counter and a plug-in called “DeadEasy Larval Glia” (Forero et al., 2012), where the threshold of detection is dependent on the brightness of Repo staining in each experiment. To make sure equal treatment of the control and experimental samples, the brains were stained in the same tube, and the experimental value is normalised to the control. As this quantification is a relative and not absolute measure of glial number, we think fold-change is the fairest way to represent this data (the raw data is presented as source data accompanying each main figure and is also presented here as Reviewer’s Fig Table 2). In contrast, NB number is counted manually and is therefore presented as raw counts.

Minor comments:

On the top of P.14, "Figure S7A-C" should probably be "Figure S6A-C"

We have corrected this, (Appendix FigS1)

Reviewer #1 (Significance (Required)):

The cell autonomous regulation of growth and proliferation of neuroblasts in the larval brain have been well-studied, but much less is known about the non-cell autonomous signals. This paper significantly moves forward knowledge in this area by describing multiple steps of a molecular mechanism for glial regulation of the neuroblast cell cycle. These findings would be of interest not only to the study of Drosophila neuroblasts, but also to the broader adult stem cell field.

My expertise is in Drosophila stem cell biology and genetics.

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

Major comments:

1.From the data in presented in Fig. 2H-K and Fig. S3C, I am very confused about role of Hh in the non-cell autonomous regulation of neuroblast cell cycle. Both RNAi and overexpression of Hh with Repo-Gal4 cause a reduction in the neuroblast EdU index (Fig. 2H-K and S3C). The authors conclude this section on p.7 saying "Together, our data suggests that high levels of glial Hh expression restricts NB cell cycle progression." This statement is not consistent with data.

With *repo-GAL4>hhRNAi*, the cortex glial niche enwrapping NBs is dramatically disrupted, which indirectly alters NB cell cycle progression, indicated by an increase in pH3 index and a decrease in EdU index. We have now included a new EdU pulse chase experiment (Fig EV3 H-K), which demonstrates that indeed glial-Hh knockdown reduces NB cell cycle progression. Both RNAi and overexpression of Hh with *repo-GAL4* causes a reduction in NB EdU index is seemingly contradictory. However, it is consistent with a previous report from Speder and Brand, 2018, where it was shown that that glial niche impairment induced by the PI3K pathway inhibition also causes a similar NB phenotype (an increase in pH3 index and a decrease in EdU incorporation). Furthermore, with *repo-GAL4>htl*^{DN}, which caused a similar glial niche impairment (data not shown), we also observed an increase in pH3 index and a slight decrease in EdU incorporation. Therefore, we concluded that the NB cell cycle progression defects upon Hh knockdown is due to cortex glial niche disruption rather than a direct effect of Hh inhibition on NBs.

What is the normal physiological role of Hh if both decreased and increased levels of cortex glial Hh expression reduce neuroblast cell cycle? The discussion of p.15 does not clarify this issue.

With regards to the physiological role of Hh, we suggest a speculative model of Hh function: low levels of glial Hh is required for the establishment/maintenance of cortex glial niche, essential to sustain NB activities, however, excessive glial Hh activates Hh signaling in the neighbouring NBs, and inhibits its cell cycle progression. In terms of how glial niche impairment impedes NB cell cycle progression, we observed that upon glial niche disruption,

NBs tend to cluster together (Fig 2F-G). Therefore, it is possible that the localization of other cell types (i.e. GMCs and neurons) are also altered as a result of NB clustering, which can potentially affect the NB cell cycle. While these questions will be interesting to explore in the future, they are beyond the scope of this current study. In contrast, we robustly showed Hh signals, when overexpressed in glial niche, were capable of making contact with NBs, resulting in stalled NB cell cycle progression. We have amended our discussion section regarding these findings (Line 443-456).

The model in Fig.7J relates to the role of Hh in the context of cortex glial FGF activation and does not illustrate the normal physiological role of Hh in the regulation of neuroblast cell cycle.
We have amended the model to include “wildtype condition” and “Glioma condition”, which is now the synopsis.

2.P.8 "Analysis of the total glial cell number indicates overexpression of htlACT, but not InRwt or EgfrACT, led to an increase in the number of cortex glial cells (Figure 4E-G, I-K)." This statement is confusing as Repo staining was used to quantify total glial numbers (including perineural, sub-perineural and cortex glia) but these data are then taken to represent and increase specifically in cortex glia. This should be clarified.

We thank the reviewer for picking this up. Our intention was to quantify the number of cortex glia cells in glial-specific htl^{ACT}, InR^{wt} and $Egfr^{ACT}$ manipulations. However, two reported cortex glial antibodies (PntP2 from Avet-Rochex et al., 2012 and SoxN described in Read, 2018), showed unspecific labelling of other cell types (Reviewer_Fig 2, arrows, neurons and NBs)[Figures for referees not shown.]. As an alternative, we quantified the total glial cell number ($Repo^{+}$) in htl^{ACT}, InR^{wt} or $Egfr^{ACT}$ overexpressed using a cortex glial driver (*NP2222-GAL4*). We expect that the alterations in glial cell number would be primarily attributed to cortex glial-specific gene manipulation. We have now changed the statement to: “we found overexpression of htl^{ACT}, but not InR^{wt} or $Egfr^{ACT}$ led to an increase in total glial cell numbers (Fig 4E-G, I-K)”. We have now clarified this in line 230-231.

3. It should be mentioned on p.8 that the data in Fig.4A-K reproduce the findings of Avet-Rochex et al., 2012 and Read et al., 2009.

We have now changed line 224-228, page 8 to "Consistent with the observation of Avet-Rochex et al. (Avet-Rochex *et al.*, 2012), we found htl^{ACT} but not InR^{wt} overexpression caused an expansion of the cortex glial niche which enwraps NBs (Fig 4 A-C'). In contrast, $Egfr^{ACT}$ overexpression, which acts through Ras signaling, didn't affect cortex glial niche size (Fig 4A-A, D-D')".

4.Figure 6F. Presumably due to the increase in glia cell number and dramatic increase in glial cell volume, any gene that is specific to, or enriched in, cortex glia will have increased expression levels in RepoGal4>htlACT larval CNS. Can the authors provide evidence that the increase in the expression of these genes is specific to FGF transcriptional regulation and not just a relative increase in the levels of these genes due to an increase in cortex glia as proportion of total CNS volume? Is there any evidence that Hh, fasn1 and lsd2 are direct transcriptional targets of FGF signalling in glia?

We agree that FGF activation causes a dramatic increase in glial cell number, thus will cause a relative increase in the level of hh, fasn1 and lsd2s. However, with RT-qPCR, the same amounts of total RNA (1µg) were extracted from control vs *repo-GAL4> htl^{ACT}* and reverse transcribed into cDNA for qPCR. Therefore, the mRNA level described in Fig 6F are already normalized to the total amount of genetic material.

In the literature, it is not reported that *hh*, *fasn1* and *lsd2* are direct transcriptional targets of FGF signalling. However, lipid metabolism rewiring is a hallmark of glioblastoma. For example, high levels of FASN has been linked with high grade glioblastoma (Grube et al., 2014). Furthermore, FGF signalling has also been shown to modulate lipid metabolism and alter the transcription of the Lsd-2 homologue called Plin2 in a mouse model (Ye et al., 2016).

To figure out whether *hh, fasn1* and *lsd2* are direct transcriptional targets of FGF signalling. we will have to first find out which TFs are altered in the glia upon altered FGF signalling via cortex glia specific RNA-seq, and then conduct DamID to identify their target genes. This would be interesting to follow-up but is beyond the scope this current study.

5. FGF signalling has been shown to be necessary and sufficient for cortex glial proliferation. So does knockdown of Htl, or expression of dominant negative Htl, cause a reduction in Hh, fasn1 and lsd2 expression in cortex glia?

In response to glial *htl^{DN}* overexpression, we observed a significant reduction in total glial number and overall Hh expression. However, RT-qPCR showed that mRNA levels of *hh, fasn1 or lsd-2* were not altered upon *htl^{DN}* overexpression (Reviewer_Fig 3)[Figures for referees not shown.].

5. Continued: If so, how does reduction of cortex glial numbers independent of FGF signalling, using for example knockdown of String or expression of Decapo, affect the expression of Hh, fasn1 and lsd2 in cortex glia?

To test this, we utilised glial specific expression of an inhibitor of the PI3K (*Δp60*), which has been shown by Speder and Brand, 2018 to cause a reduction in cortex glial number. We found that similar to htl^{DN}, reduction of cortex glia number via overexpression of *Δp60*, did not alter the expression level of *hh* or *fasn1*, however, this manipulation caused a reduction in the expression of *lsd2* (Reviewer_Fig 4)[Figures for referees not shown.].

6.Can the authors speculate on why and how increased levels of Hh in cortex glia, in the context of FGF activation, inhibit neuroblast cell cycle? Is this a physiological mechanism to limit neuroblast proliferation in the face of increased gliogenesis, or is it simply an indirect result of 'spillover' of excess Hh from cortex glia onto neuroblasts (which are autonomously regulated by Hh and so sensitive to this ligand) by due to increased cortex glia cells?

We favour the model that excess Hh in the glia compartment “spills over” to reduce NB proliferation, which are autonomously regulated by Hh and therefore are sensitive to this ligand. We have added this point to the discussion (Line 457-460).

Minor comments:

-Figure 1C' some lipid droplets are extremely large, is this consistent with previous literature?

These large lipid droplets are caused by lipid droplet fusion due to the use of detergent in this experiment. When we perform antibody staining together with lipid droplet staining, PBST detergent is required for antibody staining to work. However, this created the artefact of large lipid droplets, due to lipid droplet fusion. This has previously been reported by Bailey et al., 2015, and we have explained this in line 550-556, page 18 of the Method section.

-Including a profile plot of relative fluorescence intensity in Figure 1C',F',H' to illustrate colocalization of lipidTOX and Hh, would be helpful.
Quantification is included in Fig 1H-J.

-Figure S3A,B quantify Hh protein level and CNS size phenotypes with Hh RNAi.
Quantification is included in Fig EV3C-D.

-p.6 include data showing overexpression of Hh does not cause glial overgrowth.
Data included in Fig EV3L-O.

-Top of p.14 should be FigS6A-C.
It is now referred to as Appendix FigS1A-C.

-Include quantification of glial overgrowth and lipid droplet phenotypes with HtlACT plus catalase and SOD1 overexpression (Fig. S6D-K).
Quantification is included in Appendix FigS1G-H.

Reviewer #2 (Significance (Required)):

The is a novel and very interesting study, well written and the data are very clearly presented. It builds on and adds to the emerging literature on the glial niche and its role in neural stem cell regulation. It will be of great interest to Drosophila neurobiologists but also to the broader field of neural stem cell biology.

My expertise is Drosophila neurobiology.

Reference:

Avet-Rochex, A., Kaul, A.K., Gatt, A.P., McNeill, H., and Bateman, J.M. (2012). Concerted control of gliogenesis by InR/TOR and FGF signalling in the Drosophila post-embryonic brain. Development *139*, 2763-2772.

Bailey, A.P., Koster, G., Guillermier, C., Hirst, E.M., MacRae, J.I., Lechene, C.P., Postle, A.D., and Gould, A.P. (2015). Antioxidant Role for Lipid Droplets in a Stem Cell Niche of Drosophila. Cell *163*, 340-353.

Forero, M.G., Kato, K., and Hidalgo, A. (2012). Automatic cell counting in vivo in the larval nervous system of Drosophila. J Microsc *246*, 202-212.

Grube, S., Dunisch, P., Freitag, D., Klausnitzer, M., Sakr, Y., Walter, J., Kalff, R., and Ewald, C. (2014). Overexpression of fatty acid synthase in human gliomas correlates with the WHO tumor grade and inhibition with Orlistat reduces cell viability and triggers apoptosis. J Neurooncol *118*, 277-287.

Homem, C.C., and Knoblich, J.A. (2012). Drosophila neuroblasts: a model for stem cell biology. Development *139*, 4297-4310.

Kanai, M.I., Kim, M.J., Akiyama, T., Takemura, M., Wharton, K., O'Connor, M.B., and Nakato, H. (2018). Regulation of neuroblast proliferation by surface glia in the Drosophila larval brain. Sci Rep *8*, 3730.

Read, R.D. (2018). Pvr receptor tyrosine kinase signaling promotes post-embryonic morphogenesis, and survival of glia and neural progenitor cells in Drosophila. Development *145*.

Speder, P., and Brand, A.H. (2018). Systemic and local cues drive neural stem cell niche remodelling during neurogenesis in Drosophila. Elife *7*.

Ye, M., Lu, W., Wang, X., Wang, C., Abbruzzese, J.L., Liang, G., Li, X., and Luo, Y. (2016). FGF21-FGFR1 Coordinates Phospholipid Homeostasis, Lipid Droplet Function, and ER Stress in Obesity. Endocrinology *157*, 4754-4769.

1st Revision - Editorial Decision 10th Feb 2021

Dear Dr. Cheng

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

As you will see, both referees are very positive about the study and request only minor changes to clarify some of the results. I also noticed that the model in Fig. 7J is missing. Please include it again as it is very helpful and informative.

Browsing through the manuscript myself, I noticed a few editorial things that we need before we can proceed with the official acceptance of your study.

- We noticed that the following figure panels are never called out in the text: Fig 1H-J, Fig 4D, Fig 7H. Moreover, Fig 6F+I are called out before 6A-E. You might consider rearranging the order of the panels in this figure.

- Appendix figure S2: please specify the number of experiments (n) and the nature of the bars and error bars in the legend.

- During our routine image analysis we noticed that you show the same images in Fig. 1C and in Fig. EV2C. I understand that you highlight two different cell types in the same organ, which is as such OK. But please add a note in the respective figure legends, e.g. in that of Fig. EV2, that you show the same staining as in Fig. 1 to avoid any unambiguity.

- Table 1: Please upload this table as file type 'reagent table". It will then be typeset within the materials and methods section. See also < https://www.embopress.org/page/journal/14693178/authorguide#textformat>, Structured methods.

- I attach to this email a related manuscript file with comments by our data editors. Please address all comments and upload a revised file with tracked changes with your final manuscript submission. I have also taken the liberty to make some changes to the title and abstract. Please review these.

- The second attachment is a slightly modified version of the summary text you sent. Could you please review it and modify further if needed.

We look forward to seeing a final version of your manuscript as soon as possible.

With kind regards,

Martina

Martina Rembold, PhD
Senior Editor
EMBO reports

****

Referee #1:

In this revision, the authors have provided new data, such as the results of a EdU pulse chase experiment to confirm their conclusions about the effect of hh knockdown on the NB cell cycle; provided additional data for some experiments reported in the previous draft; and added new text and diagrams to clarify points that were confusing in the previous draft. These revisions fully address my previous concerns and I now support publication of the manuscript in its current form. I think it is important work and will have a lasting impact.

Referee #2:

I am satisfied with most of the responses to my comments and the modifications to the manuscript. However, the model that was previously included and the authors say they have modified, which should by Fig. 7J, is missing from the manuscript. It is important that this model is included.

Also, the authors have missed the point in their response to my comment (4), about whether the increase in the expression of Hh, fasn1 and lsd2 is specific to FGF transcriptional regulation and not just a relative increase in the levels of these genes due to an increase in cortex glia as proportion of total CNS volume. Regardless of the total amount of RNA used for the qRT-PCR, the fact that glia represent a far greater proportion of the cells when htlACT is expressed mean that any glial gene transcript will potentially be over-represented compared to control tissue, where there glia are a smaller proportion of the total cell number. So the fact the same amounts of total RNA were used does not address my comment. However, the data shown in Reviewer Figures 3 and 4 clearly show that in the reverse situation, when glia represent a smaller proportion of the cell population compared to control, the expression of Hh, fasn1 and lsd2 is largely unchanged. Therefore, these experiments have addressed my concerns on this point. NB, it's interesting that lds2 is decreased by deltap60 expression. Perhaps this gene is a target of the PI3K pathway?

_____

Referee #1:

In this revision, the authors have provided new data, such as the results of a EdU pulse chase experiment to confirm their conclusions about the effect of hh knockdown on the NB cell cycle; provided additional data for some experiments reported in the previous draft; and added new text and diagrams to clarify points that were confusing in the previous draft. These revisions fully address my previous concerns and I now support publication of the manuscript in its current form. I think it is important work and will have a lasting impact.

Referee #2:

I am satisfied with most of the responses to my comments and the modifications to the manuscript. However, the model that was previously included and the authors say they have modified, which should by Fig. 7J, is missing from the manuscript. It is important that this model is included.

Also, the authors have missed the point in their response to my comment (4), about whether the increase in the expression of Hh, fasn1 and lsd2 is specific to FGF transcriptional regulation and not just a relative increase in the levels of these genes due to an increase in cortex glia as proportion of total CNS volume. Regardless of the total amount of RNA used for the qRT-PCR, the fact that glia represent a far greater proportion of the cells when htlACT is expressed mean that any glial gene transcript will potentially be over-represented compared to control tissue, where there glia are a smaller proportion of the total cell number. So the fact the same amounts of total RNA were used does not address my comment. However, the data shown in Reviewer Figures 3 and 4 clearly show that in the reverse situation, when glia represent a smaller proportion of the cell population compared to control, the expression of Hh, fasn1 and lsd2 is largely unchanged. Therefore, these experiments have addressed my concerns on this point. NB, it's interesting that lds2 is decreased by deltap60 expression. Perhaps this gene is a target of the PI3K pathway?

We thank the reviewers for their insightful comments.

Reviewer #2

The model that was previously included and the authors say they have modified, which should by Fig. 7J, is missing from the manuscript. It is important that this model is included.

We have included the modified Fig 7J to the manuscript.

Also, the authors have missed the point in their response to my comment (4), about whether the increase in the expression of Hh, fasn1 and lsd2 is specific to FGF transcriptional regulation and not just a relative increase in the levels of these genes due to an increase in cortex glia as proportion of total CNS volume. Regardless of the total amount of RNA used for the qRT-PCR, the fact that glia represent a far greater proportion of the cells when htlACT is expressed mean that any glial gene transcript will potentially be over-represented compared to control tissue, where there glia are a smaller proportion of the total cell number. So the fact the same amounts of total RNA were used does not address my comment. However, the data shown in Reviewer Figures 3 and 4 clearly show that in the reverse situation, when glia represent a smaller proportion of the cell population compared to control, the expression of Hh, fasn1 and lsd2 is largely unchanged. Therefore, these experiments have addressed my concerns on this point. NB, it's interesting that lds2 is decreased by deltap60 expression. Perhaps this gene is a target of the PI3K pathway?

We thank the reviewer for the clarification and we agree that htlACT glia represents a greater proportion of the total RNA. We also agree that in htlDN, where glia represents a smaller proportion of the total RNA, Hh, fasn1 and lsd2 transcripts are largely unchanged, supporting the view that these transcripts are not simply upregulated in htlACT because glia represents a larger proportion of the total RNA. It would be interesting to examine whether lsd2 is a direct target of PI3K pathway in the future.

2nd Revision - Editorial Decision 12th Feb 2021

Dr. Louise Cheng
1 Peter MacCallum Cancer Centre, 305 Grattan St, Parkville, Victoria, 3000; 2 Sir Peter MacCallum Department of Oncology, University of Melbourne, Parkville, Victoria, Australia, 3010; 3 Department of Anatomy and Physiology, University of Melbourne, Parkville, Victoria, Australia 3010
Parkville, Victoria 3000
Australia

Dear Louise,

Thank you for incorporating all requested changes so quickly. I am now 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.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

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.

Kind regards,

Martina

Martina Rembold, PhD
Senior Editor
EMBO reports

****

THINGS TO DO NOW:

You will receive proofs by e-mail approximately 2-3 weeks after all relevant files have been sent to our Production Office; you should return your corrections within 2 days of receiving the proofs.

Please inform us if there is likely to be any difficulty in reaching you at the above address at that time. Failure to meet our deadlines may result in a delay of publication, or publication without your corrections.

All further communications concerning your paper should quote reference number EMBOR-2020-52130V3 and be addressed to emboreports@wiley.com.

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.

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: Louise Cheng
Journal Submitted to: EMBO reports
Manuscript Number: EMBOR-2020-52130V1

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 $\chi 2$ 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?	In Drosophila studies, we are not limited by animal sample size, and therefore, we generally perform n>5 for all of the experiments performed.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	N/A
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	We did not exclude any samples.
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.	We did not allocate samples to treatment. When we measure intensity of experiment vs control, we take steps to make sure the samples were stained under the same conditions, and the images were acquired in parallel under image acquisition.
For animal studies, include a statement about randomization even if no randomization was used.	Our experiments did not involve randomization.
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.	N/A
4.b. For animal studies, include a statement about blinding even if no blinding was done	No blinding was performed.
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	P-values were calculated by two-tailed, unpaired Student's t-test, with equal sample variance; The Welch's correction was applied in case of unequal variances. Kolmogorov-Smirnov test was used to test data normality. Mann-Whitney test was used when data deviated from a normal distribution.
Is there an estimate of variation within each group of data?	Yes, we assess variation for each experiment.

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?	The Welch's correction was applied in case of unequal variances

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).	Yes
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	N/A

* 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.	Yes, included in the methods.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	N/A
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.	N/A

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
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.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
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.	N/A
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.	N/A

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	Yes
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).	N/A
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).	N/A
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.	N/A

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.	N/A