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DOT1L activity affects neural stem cell division mode and reduces differentiation and ASNS expression

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Review #1

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

The manuscript investigated the role of DOT1L during neurogenesis especially focusing on the earlier commitment from APs. Using tissue culture method with single-cell tracing, they found that the inhibition of DOT1L results in delamination of APs, and promotes neuronal differentiation. Furthermore, using single cell RNA-seq, they seek possible mechanisms and changes in cellular state, and found a new cellular state as a transient state. Among differentially expressed genes, they focused on microcephaly-related genes, and found possible links between epigenetic changes led by DOT1L inhibition and epigenetic inhibition by PRC2. Based on these findings, they suggested that DOT1L could regulate neural fate commitment through epigenetic regulation. Overall, it is well written and possible links from epigenetic to metabolic regulation are interesting. However there are several issues across the manuscript.

Major issues:

1. It is not clear whether the degree of H3K79 methylation (or other histones) changes during development, and whether DOT1L is responsible for those changes. It is necessary to show the changes in histone modifications as well as the levels of DOT1L from APs to BPs and neurons, and to what extent the treatment of EPZ change the degree of histone methylation. Furthermore, the study mainly used pharmacological bath application. DOT1L has anti-mitotic effect, thus it is not clear whether the effect is coming from the inhibition of transmethylation activity. In addition, the study assumed that the effect of EPZ is cell autonomous.However, if EPZ treatment can change the metabolic state in a cell, it would be possible that observed effects was non-cell autonomous. It would be important to address if this effect is coming in a cell-autonomous manner by other means using focal shRNA-KD by IUE.
2. The possible changes in cell division and differentiation were found by very nice single-cell tracing system. However, changes in division modes occurring in targeted APs such as angles of mitotic division and the expression of mitotic markers were not addressed. These information is critical information to understand mechanisms underlying observed phenotype, delamination, differentiation and fate commitment .
3. The scRNA-seq analysis indicated interesting results, but was not fully clear to explain the observed results in histology. In fact, in single cell RNA-seq, the author claimed that cells in TTS are increased after EPZ treatment, which are more similar to APs. However, in histological data, they found that EPZ treatment increased neuronal differentiation. These data conflicts, thus I wonder whether "neurons" from histology data are actually neurons? Using several other markers simultaneously, it would be important to check the cellular state in histology upon the inhibition/KD of DOT1L.

Minor issues:

Figure 1

- It is not clear delaminated cells are APs, BPs or some transient cells (Sox2+ Tubb3+??). It is important to use several cell type-specific and cell cycle markers simulnaneously to characterize cell-type specific identity of the analysed cells by staining.These applied to Fig1B,D,E,F,G,as well as Fig2,3.

- Please provide higher magnification images of labelled cells (Fig 1H)
- Please provide clarification on the criteria of Tis21-GFP+ signal thresholding.
- Splitting the GFP signal between ventricular and abventricular does not convincingly support the "more basal and/or differentiated" states after EPZ treatment.
- Please explain the presence of Tis21-GFP+ cells at the apical VZ.
- Order the legends in same order as the bars.

Figure 2

- Fig 2B) The difference between CON and EPZ apical contacts is not clear and does not match with the graph in Fig 2E.

- Supp Fig 2 - are these injected slices cultured in control conditions? Please include this in the text and figure/figure legend

Fig 2C) The EPZ-treated DxA555+ cells exhibit morphological change of cell shape. Is this phenotype? please comment on the image shown for EPZ treatment panel.

Fig 2F - 2G) Data presented on EOMES+ and TUBB3+ % are counterintuitive. The authors claimed that TUBB3+ cells are increased and neuronal differentiation is promoted. However, no changes in EOMES+ are observed. What is the explanation? Did the author check the double positive cells? These could be TSS cells?

Figure 2 and Figure 3) the number of pairs analyzed for EPZ is twice as that of Con for comparison of the parameters taken into account. Please include n of each graph in the figure legend of the specific panel if not the same for all panels in that figure (i.e. for figure 3)

Figure 3)

- The data indicated that the number of daughter cell pairs in EPZ samples is almost double than Control. Is this the phenotype? More numbers of daughter cells in EPZ treated samples were observed from the same number of injections? or the number of injected cells were different?

Figure 4)

- Please clarify if the single cell transcriptomic analysis has been performed only once, and if yes, how statistical testing to compare the cell proportion is carried out with only one batch.

Fig 4G)

Figure 4 and 5)

- Figures are not supportive of the statement regarding APs' neurogenic potential upon DOT1L inhibition. TSS transcriptomic profile resembles more progenitors than neurons.

Please comment on TSS neurogenic capacity taking into account the provided GO and RNAseq.
- Please provide GO analysis for APs and BPs.

Figure 5)

- Reconstruct figure 5A by listing genes in the same order in both Con and EPZ, and prioritize EPZ-Con differences instead of cell-cell differences. Moreover, the presented genes in the heatmap is not the same in two conditions (i.e. NEUROG1 is present in EPZ but absent in Con). Please justify.
Fig 5D)
- Please explain why binding of EZH2 on the promoter of Asns is strongly reduced in comparison to a mild significant reduction of H3K79me/H3K27me3 in EPZ compared to Control. Also is the changed directly medicated by DOT1L? Please test whether DOT1L can bind the promoter of Asns.

Please provide the expression patterns of DOT1L and Asns during neuronal differentiation.

Other General comments:
- Please Indicate VZ, SVZ and CP on the side of the pictures/ with dot lines in the pictures both for primary figures and supplementary.
- The Results and figures sometimes do not support the statement made by the authors
- Schemes are not informative/explanatory enough, i.e. time windows of treatment and sample collection, culture conditions details..
- A more extensive characterization of TTS cells in terms of differentiation progression and integration would be enlightening
- Picture quality can be improved, provide high magnification images.

2. Significance:

Significance (Required)

The study could be important for the specific field in neural development.
It aims to understand mutations in respective genes and brain malformation.
If the link between epigenetic and metabolic changes is clearly shown, it will be interesting. However, the current manuscript is still rather descriptive, and clear mechanistic insights were not provided. The study have potentials and additional data will strength the value of study.

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 3 and 6 months

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Review #2

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

Appiah et al. present a concise manuscript that provides details and possible mechanisms of their previous work (Franz et al., 2019; Ferrari et al., 2020). The study uses diverse lines of investigation to arrive at most conclusions. However, as interesting as the data is, we find that at the present state, it is not sufficient to prove that, indeed, the asparagine metabolism is regulated by DOTL1/PRC2 crosstalk.
The neurogenic shift presented in the first part of the paper is not comprehensive and, therefore, not very convincing. The quality of images provided in the main and supplementary data is less than ideal.
Additional data analysis and interpretation of the scRNA seq data may be needed.
The authors finally conclude with rescue experiments done in culture and in-vivo, which we believe is the stand-out part of this study.
Overall the manuscript has some interesting observations that are often over-interpreted with less supporting data. The manuscript reads well but requires additional data and changes in the claims/interpretation to be suited for publication.

Comments

1. Abstract:
Is this statement correct: "DOT1L inhibition led to increased neurogenesis driven by a shift from asymmetric self-renewing to symmetric neurogenic divisions of APs". AP undergoes symmetric division for self-renewal and asymmetric neurogenic divisions.

All the data is based on treatments with EPZ (DOTL1 inhibitor), yet no information is shown to support its targeted activity in this system. A proof of principle in the chosen

experimental system is missing; for instance, examining the activity or protein level of DOTL1 and decreased methylation of the target(s) is essential.
2. Figure 1: The scoring of centrosomes and cilia is insufficient to conclude delamination and increase in basal fates. The effect could be on ciliogenesis or centrosome tethering to the apical end-feet of the AP, and other possible explanations for this observation also exist. The images are too small; larger images or graphic representations could be helpful in addition to the data.

To make a statement regarding delamination, I would like to see either the dynamics of delamination (organotypic slices images), staining with BP markers, or morphological changes of AP (staining that will reveal loss of adherence) or comparable data to support the observation. In my opinion Supp. Figure 1 is insufficient; the single image is not convincing; I would like to see 3D reconstruction and better quality images.

Tis21 data (1H), again of low quality, is only a single piece of evidence and the conclusion "suggesting that the acquisition of a basal fate was paralleled by a switch to neurogenesis" is premature. I think other cell cycle exit reporters, Fucci markers, pHis, BrdU, NeuroD, or Tbr2 reporters (Li et al., 2020, (Haydar and Sestan labs)) to name a few, are necessary to establish the conclusions. The authors should show other markers such as PAX6, EOMES, or other upper-layer markers upon cell cycle exit in the SVZ/CP. These additional experiments will assist in cell fate analysis.
2. Figure 2: The microinjection experiments are elegant; the images, however, do not complement the experiment. The images of the microinjected cells seem not to be reconstructed from z-stacked optical slices, so often, processes are not continuous (panel B, for example); therefore, it is not clear if an apical process is indeed missing or just not seen. The data analysis should include other parameters; BrdU staining could have given information on cell cycle exit, PAX6, SOX2, and EOMES on the location of the cells in the VZ/sVZ. The quality of images showing EOMES and TUBB3 staining is so low that it makes the reader doubt the validity of the quantifications.
"Taken together, these data suggest that the inhibition of DOT1L might favor the acquisition of a neuronal over BP cell fate" This interpretation should be subjected to more investigations. It is possible that this treatment just accelerates the AP-> BP -> Neuronal fate.
The author's claim needs to be backed by additional experiments or be changed.
3. Figure 3: The experiment concept and its performance are impressive, yet the data is insufficient. The images in A that are supposed to be representative show two cells; their location is not clear, and the expression of GFP is not clear; in fact, both pairs seem to be GFP negative (not clear what is the threshold for background). Staining with anti-GFP and a second method to follow neurogenesis is necessary.
4. On page 9, lines 8-10, the authors claim that their number of cells was "sufficient" for single-cell analysis; the numbers are <500 for all samples. The authors need to justify this statement or articles that carefully analyze the number required for such a conclusion as references.
5. The authors use Seurat and RaceID without their appropriate citations in the first mention during the results. The authors also stop immediately after DEG analysis along with clustering. The authors could analyze their RNA-seq data with a trajectory; to say the least, the identification/characterization of TTS and neurons as Neurons I, II, and III are insufficient. There could be multiple ways to show the "fate" of cells in the isolated FACS, which the authors have missed.

6. The authors detected candidates like Fgfr3, Nr2f1, Ofd1, and Mme as part of their treated (different approaches) datasets (from their DEG analysis). They correctly cite Huang et al., 2020 but fail to give us a sense of the consequences of these gene dysregulations. The authors can also validate if these proteins are expressed in their treated cells.
7. The authors list a few GO terms (page 10, lines 1-10) and associate them with reduced proliferation; they must cite relevant studies. The authors can also add supplementary data showing which genes in their data correspond to these GO terms.
8. On Page 11, lines 3-7, the authors describe their method to arrive at the 17 targets with TF activity from the previous analysis. Can the authors describe the method used to correlate the two? The reviewer understands this could be MEME analysis or analysis of earlier datasets of Ferrari et al. 2020. But it must be explicitly stated, and a few examples in supplementary need to be exemplified as this analysis is key to discovering the three metabolic genes.

2. Significance:

Significance (Required)

Appiah et al. present a concise manuscript that provides details and possible mechanisms of their previous work (Franz et al., 2019; Ferrari et al., 2020). The study uses diverse lines of investigation to arrive at most conclusions. However, as interesting as the data is, we find that at the present state, it is not sufficient to prove that, indeed, the asparagine metabolism is regulated by DOTL1/PRC2 crosstalk.
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The authors finally conclude with rescue experiments done in culture and in-vivo, which we believe is the stand-out part of this study.

Overall the manuscript has some interesting observations that are often over-interpreted with less supporting data. The manuscript reads well but requires additional data and changes in the claims/interpretation to be suited for publication.

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

More than 6 months

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Reviewer Publons

Yes

Revision Plan

Manuscript number: RC-2022-01528
Corresponding author(s): Elena Taverna and Tanja Vogel

1. General Statements [optional]

We thank the reviewers for the comments and points they raised. We think what we have been asked is a doable task for us. The present revision plan is set to address all points in a manner that we hope the reviewer will find satisfactory.

The title of the manuscript was changed to better fit and describe the data we present.

2. Description of the planned revisions

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

Reviewer's comment:
The manuscript investigated the role of DOT1L during neurogenesis especially focusing on the earlier commitment from APs. Using tissue culture method with single-cell tracing, they found that the inhibition of DOT1L results in delamination of APs, and promotes neuronal differentiation. Furthermore, using single cell RNA-seq, they seek possible mechanisms and changes in cellular state, and found a new cellular state as a transient state. Among differentially expressed genes, they focused on microcephaly-related genes, and found possible links between epigenetic changes led by DOT1L inhibition and epigenetic inhibition by PRC2. Based on these findings, they suggested that DOT1L could regulate neural fate commitment through epigenetic regulation. Overall, it is well written and possible links from epigenetic to metabolic regulation are interesting. However, there are several issues across the manuscript.
Response to Reviewer and planned revision:
We thank the reviewer's 1 for her/his comments and constructive criticism.
We hope the revision plan will address the points raised by the reviewer in a satisfactory manner.

Major issues:

Reviewer's comment:
1) It is not clear whether the degree of H3K79 methylation (or other histones) changes during development, and whether DOT1L is responsible for those changes. It is necessary to show the changes in histone modifications as well as the levels of DOT1L from APs to BPs and neurons, and to what extent the treatment of EPZ change the degree of histone methylation.
Response to Reviewer and planned revision:

- As for the level of DOT1L protein

We tried several commercially available antibodies, but they do not work in the mouse, even after multiple attempts and optimization. So, unfortunately we will not be able to provide this piece of information.

- As for the level of DOT1L mRNA
  We can provide info regarding the DOT1L mRNA level in APs, BPs and neurons by using scRNAseq data from E12, E14, E16 WT cerebral cortex.
- As for the levels of H3K79methylation, we thank the reviewer for raising this point, that is certainly a crucial one. To address the point raised by the reviewer we envisage 2 options , listed here in order of priority and ease of execution from our side.
  - immunofluorescence with an Ab against H3K79me2 using CON and EPZ-treated hemispheres
    and/or
  - FACS sort APs, BPs and neurons from CON and EPZ-treated hemispheres, followed by immunoblot for H3K79me2 to assess the H3K79me2 levels. As for the FACS sorting, we will use a combinatorial sorting in the lab on either a TUBB3-GFP or a GFP-reporter line using EOMES-driven mouse lines. This strategy has already been employed in the lab by Florio et al., 2015 and we will use it with minor modifications.

Reviewer's comment:
Furthermore, the study mainly used pharmacological bath application. DOT1L has anti-mitotic effect, thus it is not clear whether the effect is coming from the inhibition of transmethylation activity.
Response to Reviewer and planned revision:
We are aware of the potential pitfalls associated with the use of a pharmacological treatment. We would like here to clarify the reason we decioded to use this experimental paradigm.
Our previous work on DOT1L made use of a genetic model (DOT1L conditional KO mouse, Franz et al. 2019).
Although powerful, the genetic model relying on the full ablation of DOT1L did not allow us to understand if the role of DOT1L in brain development is mediated by its scaffolding or enzymatic activity. We wanted to fill this gap in knowledge by focusing specifically on DOT1L's enzymatic activity. This is the reason why we choose to focus on the pharmacological inhibition with EPZ, whose effect on DOT1L activity has been extensively reported and documented in literature (EPZ is currently in phase clinical 3 studies)
We might have not justified in a convincing way our rationale in the text, so we will further discuss and elaborate on that in the revised manuscript.

As for the anti-mitotic effect of DOT1L. This explanation cannot be excluded and should therefore be tested.
We can experimentally test the hypothesis by measuring the number of mitotic APs and BPs in control and treated hemispheres. We will use the standard approach in the field, namely the staining with anti-PH3 (see also below).

Revision Plan

Reviewer's comment:
In addition, the study assumed that the effect of EPZ is cell autonomous. However, if EPZ treatment can change the metabolic state in a cell, it would be possible that observed effects was non-cell autonomous. It would be important to address if this effect is coming in a cell-autonomous manner by other means using focal shRNA-KD by IUE.
Response to Reviewer and planned revision:
We are open on the possibility of a cell autonomous vs non-cell autonomous effect of EPZ. Indeed, we consider both explanations to be potentially valid and it is entirely possible that the effects are non-cell autonomous. We will certainly comment and elaborate on that in the revised manuscript.
To address this point exerimentally, we can proceed as follows:

- DOT1L shRNA-KD via in utero electroporation, followed by either
  - (i) in situ hybridization for ASNS to check if ASNS transcript is increased upon DOT1L shRNA-KD compared to CON
  - (ii) FACS sorting of the positive electroporated cells (CON and DOT1L shRNA-KD), followed by qPCR to assess the levels of ASNS
  - (iii) If the reviewer wants us to check for a more downstream effect on fate, then we will immuno-stain the DOT1L shRNA-KD and CON with TUBB3 AB and/or TBR1 AB (as already done in the present version of the manuscript).

We hope the reviewer will find our experimental strategy satisfactory.
We nevertheless would like to point out that shRNA-mediated KD approaches interfere with the presence of DOT1L protein and goes beyond our experimental paradigm investigating mainly the effects upon DOT1L activity inhibition - a logic we were coherently applying throughout our present manuscript (see also the comment above). It might be possible that we are not observing exactly the same effects, if loss of the protein has broader consequences which might cover effects of isolated enzymatic inhibition. This experimental approach, despite being interesting in itself, might therefore result in non-coherent observations.

Reviewer's comment:
2) The possible changes in cell division and differentiation were found by very nice single-cell tracing system. However, changes in division modes occurring in targeted APs such as angles of mitotic division and the expression of mitotic markers were not addressed. This information is critical information to understand mechanisms underlying observed phenotype, delamination, differentiation and fate commitment.
Response to Reviewer and planned revision:
We thank the reviewer for raising this point. Indeed, in a previous work using DOT1L conditional KO mouse (Franz et al. 2019), we observed a change in the mitotic spindle orientation.
We can certainly address this point by quantifying the spindle angle in CON and EPZ-treated cortical hemispheres.
From a practical point of view, we will co-stain for DAPI and phalloidin (filamentous actin counterstain) to precisely visualize DNA/chromosomes, the apical surface and the cell contour.

Revision Plan

This protocol is already established in the lab based on published work from the Huttner lab (Taverna et al, 2012; Kosodo et al, 2005).

Reviewer's comment:
3) The scRNA-seq analysis indicated interesting results, but was not fully clear to explain the observed results in histology. In fact, in single cell RNA-seq, the author claimed that cells in TTS are increased after EPZ treatment, which are more similar to APs. However, in histological data, they found that EPZ treatment increased neuronal differentiation. These data conflicts, thus I wonder whether "neurons" from histology data are actually neurons? Using several other markers simultaneously, it would be important to check the cellular state in histology upon the inhibition/KD of DOT1L.
Response to Reviewer and planned revision:
We indeed found that TTS cells are an intermediate state between APs and neurons in term of transcriptional profile (for this reason we called this cell cluster transient transcriptional state). We will address the point raised by the reviewer by performing the following immunofluorescence co-staining:

- TBR1 and/or CTIP2 in CON and EPZ-treated hemispheres
- EOMES and SOX2 in CON and EPZ-treated hemispheres

Minor issues:

Reviewer's comment:
Figure 1
- It is not clear delaminated cells are APs, BPs or some transient cells (Sox2+ Tubb3+??). It is important to use several cell type-specific and cell cycle markers simultaneously to characterize cell-type specific identity of the analysed cells by staining. These applied to Fig1B,D,E,F,G,as well as Fig2,3.
Response to Reviewer and planned revision:
We agree with the reviewer and will address this point by using a combinatorial staining scheme for several fate markers such as TUBB3, EOMES and SOX2, as suggested by the reviewer.

Reviewer's comment:
- Please provide higher magnification images of labelled cells (Fig 1H)
Response to Reviewer and planned revision:
In the revised manuscript, we will provide higher magnification for the staining.

Reviewer's comment:
- Please provide clarification on the criteria of Tis21-GFP+ signal thresholding.
Response to Reviewer and planned revision:
In the revised manuscript, we will provide a clarification on the criteria of Tis21-GFP+ signal thresholding.

Revision Plan

Reviewer's comment:
- Splitting the GFP signal between ventricular and abventricular does not convincingly support the "more basal and/or differentiated" states after EPZ treatment.
Response to Reviewer and planned revision:
We will provide a clarification regarding this point.

Reviewer's comment:
- Please explain the presence of Tis21-GFP+ cells at the apical VZ.
Response to Reviewer and planned revision:
Tis21-GFP+ cells at the apical VZ have been extensively reported in the literature, since the first paper by Haubensak et al. regarding the generation of the Tis21-GFP+ line.
In a nutshell, Tis21-GFP+ cells are present throughout the VZ (and also in the apical portion) because neurogenic, Tis21-GFP positive cells are undergoing mitosis at the apical surface. Indeed, the presence of Tis-21 GFP signal have been extensively used by the Huttner lab and collaborators to score apical neurogenic mitosis.
In addition and related to the previous point, Tis21-GFP+ nuclei are going to be present throughout the entire VZ because AP undergo interkinetic nuclear migration,.
In the revised manuscript, we will explain this point and cite additional literature for clarity.

Reviewer's comment:
- Order the legends in same order as the bars.
Response to Reviewer and planned revision:
We will follow reviewers' recommendation and order the legends accordingly.

Reviewer's comment:
Figure 2
-Fig 2B) The difference between CON and EPZ apical contacts is not clear and does not match with the graph in Fig 2E.
Response to Reviewer and planned revision:
We will explain Fig. 2B in more detail and provide additional images in the revised manuscript.

Reviewer's comment:
-Supp Fig 2 - are these injected slices cultured in control conditions? Please include this in the text and figure/figure legend
Response to Reviewer and planned revision:
In the revised manuscript, the text will be changed to address this point and provide clearer info.

Reviewer's comment:
Fig 2C) The EPZ-treated DxA555+ cells exhibit morphological change of cell shape. Is this phenotype? please comment on the image shown for EPZ treatment panel.
Response to Reviewer and planned revision:
We thank the reviewer for raising this very interesting point. We think that the change in morphology might be a consequence of delamination and/or of cell fate, as the two processes

are strictly intermingled even in physiological conditions. In the revised manuscript, we will certainly better comment on this very relevant point and expand the discussion accordingly.

Reviewer's comment:
Fig 2F - 2G) Data presented on EOMES+ and TUBB3+ % are counterintuitive. The authors claimed that TUBB3+ cells are increased and neuronal differentiation is promoted. However, no changes in EOMES+ are observed. What is the explanation? Did the author check the double positive cells? These could be TSS cells?
Response to Reviewer and planned revision:
We thank the reviewer for raising this point.
As suggested by the reviewer, we suspect that the counterintuitive data might be due to TSS cell, which based on our scRNAseq data are expressing at the same time several cell type specific markers. It is possible that, since the treatment with EPZ is 24h long, cells (like the TTS cluster) have no time to eliminate the EOMES protein. If that were to be the case, then we would expect to still detect (as we indeed do) EOMES immunoreactivity.
To address this point, we will:

- analyze scRNA-seq data and check which is the extent of co-expression of *Eomes* and *Tubb3* mRNAs in the TTS population.
- Check for EOMES and TUBB3 double positive cells in the microinjection experiment.

Reviewer's comment:
Figure 2 and Figure 3) the number of pairs analyzed for EPZ is twice as that of Con for comparison of the parameters taken into account. Please include n of each graph in the figure legend of the specific panel if not the same for all panels in that figure (i.e. for figure 3)
Response to Reviewer and planned revision:
We will revise the legend text accordingly.

Reviewer's comment:
Figure 3)
The data indicated that the number of daughter cell pairs in EPZ samples is almost double than Control. Is this the phenotype? More numbers of daughter cells in EPZ treated samples were observed from the same number of injections? or the number of injected cells were different?
Response to Reviewer and planned revision:
Due to technical reasons, we performed a higher number of injections in EPZ-treated slices. We think this is the main reason behind the difference in number.
If the reason were to be biological, one would expect to see the same trend in IUE experiments, but this is actually not the case. This does suggest/corroborate the idea that the reason behind the difference in microinjection experiments is mainly technical.

Reviewer's comment:
Figure 4)

- Please clarify if the single cell transcriptomic analysis has been performed only once, and if yes, how statistical testing to compare the cell proportion is carried out with only one batch. Fig 4G)

Response to Reviewer and planned revision:

As for the scRNAseq on microinjected cells:
the scRNA-seq analysis was done once using cells pooled from 3 different microinjection experiments performed in 3 different days.
As for the scRNAseq on IUE cells:
The scRNA-seq analysis was done once using cells pooled from 2-3 different IUE experiments performed in 3 different days.
For all scRNAseq experiments the statistical testing is achieved by intrasample comparisons according to established bioinformatics pipelines.
We will better explain this point in the revised manuscript.

Reviewer's comment:

Figure 4 and 5)
- Figures are not supportive of the statement regarding APs' neurogenic potential upon DOT1L inhibition. TSS transcriptomic profile resembles more progenitors than neurons. Please comment on TSS neurogenic capacity taking into account the provided GO and RNAseq.

Response to Reviewer and planned revision:

We thank Reviewer 1 for raising this point. It is indeed true that although TTS have a mixed signature, they do resemble more AP than neurons (as indicated in the Fig. S5B, C). We interpreted this finding that these cells are transient and therefore still maintain some AP features. Interestingly, TTS downregulate cell division markers, suggesting a restriction of proliferative potential, as one would expect for cells with an increased neurogenic potential. Following the reviewer's comment, we will discuss this point in the revised manuscript.

Reviewer's comment:

- Please provide GO analysis for APs and BPs.

Response to Reviewer and planned revision:

Following the reviewer's suggestion, we will incorporate a more careful and in-depth analysis in the revised version of the manuscript.

Reviewer's comment:

- Reconstruct figure 5A by listing genes in the same order in both Con and EPZ and prioritize EPZ-Con differences instead of cell-cell differences.

Response to Reviewer and planned revision:

We will revise Figure 5A based on the reviewer's comment.

Reviewer's comment:

Moreover, the presented genes in the heatmap is not the same in two conditions (i.e. NEUROG1 is present in EPZ but absent in Con). Please justify.

Response to Reviewer and planned revision:

Revision Plan

This observation is based on different activities of transcription factor networks in the control and EPZ condition. They are not necessarily supposed to be the same as the cell states are altered and different TF are expressed and active upon the treatment in the diverse cell types. In a revised manuscript we will justify and develop this point.

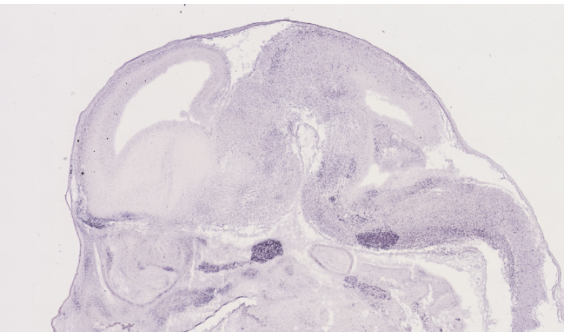
Reviewer's comment:
Fig 5D)
- Please explain why binding of EZH2 on the promoter of Asns is strongly reduced in comparison to a mild significant reduction of H3K79me/H3K27me3 in EPZ compared to Control.
Response to Reviewer and planned revision:
We can envisage several explanations
First, the simpler explanation could be that the variation is due to batch effects.
Second, the acute reduction of EZH2 might not be directly accompanied by a reduced histone mark, which is reduced either by cell division or by demethylases. The two processes of getting rid of the mark might be slower than the reduction of EZH2 presence at the respective site.
Considering the reviewer's comment, we will explain and better discuss this point in the revised manuscript.

Reviewer's comment:
Also is the changed directly medicated by DOT1L?
Please test whether DOT1L can bind the promoter of Asns.
Response to Reviewer and planned revision:
To address this relevant issue, we will proceed with the following protocol:

(i) electroporate a tagged version of DOT1L into ESCs
(ii) select ESCs and differentiate them into NPC_48h.
(iii) treat NPC with DMSO (Con) or EPZ
(iv) harvest CON and EPZ-treated NPC
(v) perform ChIP-qPCR DOT1L at the Asns promoter

Reviewer's comment:
Please provide the expression patterns of DOT1L and Asns during neuronal differentiation.
Response to Reviewer and planned revision:
As for *Dot1l*
Dot1l expression was shown in Franz et al 2019, by ISH from E12.5 to E18.5.
As for *Asns*
We will provide E14.5 in situ staining of *Asns* in the developing mouse brain using the Gene Paint database (see Figure below).
If the antibody against ASNS works in the mouse, we will also show immunostainings for ASNS at mid-neurogenesis, E14.5, which is the relevant time window for our analysis.

Revision Plan



Other General comments:

Reviewer's comment:
Please Indicate VZ, SVZ and CP on the side of the pictures/ with dot lines in the pictures both for primary figures and supplementary.
Response to Reviewer and planned revision:
The figures will be revised based on the reviewer's comment.

Reviewer's comment:
- The Results and figures sometimes do not support the statement made by the authors
Response to Reviewer and planned revision:
We thanks the reviewer for raising this point: we will carefully check on this and eliminate any overinterpretation or non-supported statements from the text.

- Schemes are not informative/explanatory enough, i.e. time windows of treatment and sample collection, culture conditions details.
Response to Reviewer and planned revision:
We will revise the schemes to include more details.
In particular, we will add a supplementary figure with a detailed visual description of the protocol, to match the description presented in the materials and methods.

Reviewer's comment:
- A more extensive characterization of TTS cells in terms of differentiation progression and integration would be enlightening
Response to Reviewer and planned revision:
We thank the reviewer for pointing her/his attention on the TTS cell cluster.
Although the TTS population is intriguing, our ability to carry out an in-depth analysis of this cell state is limited. This is because of two reasons. One is the lack of a specific marker gene for TTS, the other is the relatively small size of the TTS subpopulation.
To meet the reviewer's comment and expectation, we will use RNA velocity trajectory to expand the analysis and characterization of the differentiation potential of TTS. In the revised manuscript, we will expand the discussion accordingly.

Revision Plan

Reviewer's comment:
- Picture quality can be improved, provide high magnification images.
Response to Reviewer and planned revision:
We will revise the figures to include higher magnification images.

Reviewer #1 (Significance (Required)):

Reviewer's comment:
The study could be important for the specific field in neural development.
It aims to understand mutations in respective genes and brain malformation.
If the link between epigenetic and metabolic changes is clearly shown, it will be interesting.
However, the current manuscript is still rather descriptive, and clear mechanistic insights were not provided. The study have potentials and additional data will strength the value of study.
Response to Reviewer and planned revision:
We thank the reviewer for the constructive comments and criticism.
During the revision we will address the direct impact of DOT1L and H3K79me2 on the *Asns* gene locus (see the rationale of the experimental strategy also in the revision plan above).
By doing so, we hope we will strengthen further the mechanistic link between epigenetics and altered metabolome.

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

Reviewer's comment:
Appiah et al. present a concise manuscript that provides details and possible mechanisms of their previous work (Franz et al., 2019; Ferrari et al., 2020). The study uses diverse lines of investigation to arrive at most conclusions. However, as interesting as the data is, we find that at the present state, it is not sufficient to prove that, indeed, the asparagine metabolism is regulated by DOTL1/PRC2 crosstalk.
The neurogenic shift presented in the first part of the paper is not comprehensive and, therefore, not very convincing. The quality of images provided in the main and supplementary data is less than ideal.
Additional data analysis and interpretation of the scRNA seq data may be needed.
The authors finally conclude with rescue experiments done in culture and in-vivo, which we believe is the stand-out part of this study.
Overall the manuscript has some interesting observations that are often over-interpreted with less supporting data. The manuscript reads well but requires additional data and changes in the claims/interpretation to be suited for publication.
Response to Reviewer and planned revision:
We thank the reviewer for the constructive comments and criticism. Based on her/his comments, we designed an experimental plan that we hope will address comments and concerns in a satisfactory manner.

Revision Plan

Comments

Reviewer's comment:
1) Abstract:
Is this statement correct: "DOT1L inhibition led to increased neurogenesis driven by a shift from asymmetric self-renewing to symmetric neurogenic divisions of APs. AP undergoes symmetric division for self-renewal and asymmetric neurogenic divisions.
Response to Reviewer and planned revision:
Based on the current literature (cit. Huttner and Kriegstein), AP undergo:

- symmetric division for proliferative division *at early stages of neurogenesis*
- asymmetric self-renewing division, generating an AP and a BP *at mid neurogenesis*. This division is also described as neurogenic, as it produces a BP, that is a step further than AP in term of neurogenic potential.
- symmetric consumptive division *at late neurogenesis*

To avoid any possible confusion, we will re-phrase the sentence to include the adjective "consumptive" and specify the composition of the progeny.
In the revised manuscript, the sentence will read as follow:
"DOT1L inhibition led to increased neurogenesis driven by a shift of APs from asymmetric self-renewing (generating one AP and one BP) to symmetric consumptive divisions (generating two neurons)"

Reviewer's comment:
All the data is based on treatments with EPZ (DOTL1 inhibitor), yet no information is shown to support its targeted activity in this system. A proof of principle in the chosen experimental system is missing; for instance, examining the activity or protein level of DOTL1 and decreased methylation of the target(s) is essential.
Response to Reviewer and planned revision:
We agree with the points raised by the referee and we plan to it by assessing the methylation state upon EPZ treatment (see below).
In general, EPZ is a well characterized drug, that has been used previously in our lab and by others as well. As for the Vogel lab, the information regarding EPZ , its activity and efficiency in inhibiting DOT1L towards H3K79me2 was shown in Franz et al. 2019, Supplementary Fig. S6 D, E.
In the present manuscript, an additional confirmation that EPZ targets DOT1L in regard to its H3K79me2 activity is shown in Fig. 5D. We will refer to this information more explicitly in a revised manuscript.
In addition, we will directly address the question by extending the analysis of the methylation state, as illustrated previously.
We envisage 2 options, listed here in order of priority and ease of execution from our side.

- immunofluorescence with an Ab against H3K79me2 using CON and EPZ-treated hemispheres
  and/or

- FACS sort APs, BPs and neurons from CON and EPZ-treated hemispheres, followed by immunoblot for H3K79me2 to assess the H3K79me2 levels. As for the FACS sorting, we will use a combinatorial sorting in the lab on either a TUBB3-GFP or a GFP-reporter line using EOMES-driven mouse lines. This strategy has already been employed in the lab by Florio et al., 2015 and we will use it with minor modifications."

Reviewer's comment:
2) Figure 1: The scoring of centrosomes and cilia is insufficient to conclude delamination and increase in basal fates. The effect could be on ciliogenesis or centrosome tethering to the apical end-feet of the AP, and other possible explanations for this observation also exist. The images are too small; larger images or graphic representations could be helpful in addition to the data.
Response to Reviewer and planned revision:
We agree with the reviewer's comment. We in fact did not want to claim that the change in centrosome location *demonstrate* delamination, but only that it *suggests* delamination.
To avoid any misunderstanding regarding our interpretation of that data, we will re-phrase in a more cautious way the text referring to Figure 1 to highlight that the data *only suggest* delamination.
We also plan to better explain in the material and methods our approach in the context of the present literature. Indeed, the relocation of the centrosomes has been extensively used as a proxy for delamination by several labs working on the cell biology of neurogenesis, such Huttner and Gotz labs. We will therefore refer to this work more clearly in the revised manuscript.

Response to Reviewer and planned revision:
To make a statement regarding delamination, I would like to see either the dynamics of delamination (organotypic slices images), staining with BP markers, or morphological changes of AP (staining that will reveal loss of adherence) or comparable data to support the observation. In my opinion Supp. Figure 1 is insufficient; the single image is not convincing; I would like to see 3D reconstruction and better-quality images.
Response to Reviewer and planned revision:
We thank the reviewer for his/her comment. We can certainly provide better images, to address the morphological stainings of AP, and co-stain with relevant markers, to address the visualization of BPs. We think it is beyond the scope of the manuscript embarking in live imaging as we are not studying the dynamics of delamination *per se*.

Reviewer's comment:
Tis21 data (1H), again of low quality, is only a single piece of evidence and the conclusion "suggesting that the acquisition of a basal fate was paralleled by a switch to neurogenesis" is premature. I think other cell cycle exit reporters, Fucci markers, pHis, BrdU, NeuroD, or Tbr2 reporters (Li et al., 2020, (Haydar and Sestan labs)) to name a few, are necessary to establish the conclusions. The authors should show other markers such as PAX6, EOMES, or other upper-layer markers upon cell cycle exit in the SVZ/CP. These additional experiments will assist in cell fate analysis.

Revision Plan

Response to Reviewer and planned revision:
We completely understand the points raised by the reviewer, and we will address them by co-staining with PAX6/SOX2, PH3 and/or EOMES.
We think establishing the Fucci or EOMES mouse system is beyond the scope of the manuscript. In addition, given the present setting of all labs involved, it would be logistically unattainable (see also comments in the section below).
We think the co-staining scheme and plan will be informative to satisfactory address the concerns raised by the reviewer.

Reviewer's comment:
2) Figure 2: The microinjection experiments are elegant; the images, however, do not complement the experiment. The images of the microinjected cells seem not to be reconstructed from z-stacked optical slices, so often, processes are not continuous (panel B, for example); therefore, it is not clear if an apical process is indeed missing or just not seen.
Response to Reviewer and planned revision:
The mentioned images are reconstructed from continuous Z-stacks, as we always do given the type of data. We can provide better reconstructions and/or additional images to address the concerns raised by the reviewer.

Reviewer's comment:
The data analysis should include other parameters; BrdU staining could have given information on cell cycle exit, PAX6, SOX2, and EOMES on the location of the cells in the VZ/sVZ. The quality of images showing EOMES and TUBB3 staining is so low that it makes the reader doubt the validity of the quantifications.
"Taken together, these data suggest that the inhibition of DOT1L might favor the acquisition of a neuronal over BP cell fate" This interpretation should be subjected to more investigations. It is possible that this treatment just accelerates the AP-> BP -> Neuronal fate.
The author's claim needs to be backed by additional experiments or be changed.
Response to Reviewer and planned revision:
To address this valid concern of our reviewer, we will include in the revised manuscript staining and co-staining with PAX6, SOX2 (see also response above) and provide a BrdU labeling experiment.

Reviewer's comment:
3) Figure 3: The experiment concept and its performance are impressive, yet the data is insufficient. The images in A that are supposed to be representative show two cells; their location is not clear, and the expression of GFP is not clear; in fact, both pairs seem to be GFP negative (not clear what is the threshold for background). Staining with anti-GFP and a second method to follow neurogenesis is necessary.
Response to Reviewer and planned revision:
We will deepen our analysis by using additional markers, such as TBR1 to expand the staining schemes to follow neurogenesis.

Reviewer's comment:
4) On page 9, lines 8-10, the authors claim that their number of cells was "sufficient" for single-cell analysis; the numbers are <500 for all samples. The authors need to justify this statement or articles that carefully analyze the number required for such a conclusion as references.
Response to Reviewer and planned revision:
In the revised manuscript, we will include the analysis of how many cells are needed to identify cluster of 6 cell types in this paradigm, based for example on the algorithms developed in our collaborative efforts (see Treppner et al. 2021).

Reviewer's comment:
5) The authors use Seurat and RaceID without their appropriate citations in the first mention during the results. The authors also stop immediately after DEG analysis along with clustering. The authors could analyze their RNA-seq data with a trajectory; to say the least, the identification/characterization of TTS and neurons as Neurons I, II, and III are insufficient. There could be multiple ways to show the "fate" of cells in the isolated FACS, which the authors have missed.
Response to Reviewer and planned revision:
Based on the reviewer's comment, we will include the respective citations in a revised manuscript. We already provide differentiation trajectories but will include other methods, such as scVelo of FateID to extend the trajectory analyses.
We kindly ask the reviewer to also refer to the comments above regarding the TTS cluster characterization as part of our effort to provide a better picture of the different clusters.

Reviewer's comment:
6) The authors detected candidates like Fgfr3, Nr2f1, Ofd1, and Mme as part of their treated (different approaches) datasets (from their DEG analysis). They correctly cite Huang et al., 2020 but fail to give us a sense of the consequences of these gene dysregulations. The authors can also validate if these proteins are expressed in their treated cells.
Response to Reviewer and planned revision:
In the revised manuscript we will comment on the function of the four genes mentioned.
In addition, we will validate the expression of these genes on protein and transcriptional level through immunostainings -provided that antibodies are working in our system- or smFISH, respectively.

Reviewer's comment:
7) The authors list a few GO terms (page 10, lines 1-10) and associate them with reduced proliferation; they must cite relevant studies. The authors can also add supplementary data showing which genes in their data correspond to these GO terms.
Response to Reviewer and planned revision:
We thank the reviewer for pointing out the missing citations. We of course agree on the need to add them, and we will do so in the revised manuscript. We will also provide information about the specific genes that associate with the respective GO terms as addition to the supplementary

data.

Reviewer's comment:
8) On Page 11, lines 3-7, the authors describe their method to arrive at the 17 targets with TF activity from the previous analysis. Can the authors describe the method used to correlate the two? The reviewer understands this could be MEME analysis or analysis of earlier datasets of Ferrari et al. 2020. But it must be explicitly stated, and a few examples in supplementary need to be exemplified as this analysis is key to discovering the three metabolic genes.
Response to Reviewer and planned revision:
In the revised manuscript, we will clarify the exact analysis that resulted in the identification of the 17 target genes, using the specific tool for gene network analysis, that is based on our scRNA-seq data alone, but not on the Ferrari et al 2020 data set.

3. Description of the revisions that have already been incorporated in the transferred manuscript

n/a

4. Description of analyses that authors prefer not to carry out

Reviewer's comment:
Tis21 data (1H), again of low quality, is only a single piece of evidence and the conclusion "suggesting that the acquisition of a basal fate was paralleled by a switch to neurogenesis" is premature. I think other cell cycle exit reporters, Fucci markers, pHis, BrdU, NeuroD, or Tbr2 reporters (Li et al., 2020, (Haydar and Sestan labs)) to name a few, are necessary to establish the conclusions. The authors should show other markers such as PAX6, EOMES, or other upper-layer markers upon cell cycle exit in the SVZ/CP. These additional experiments will assist in cell fate analysis.
Response to Reviewer and planned revision:
As pointed out above, we think establishing the Fucci or EOMES mice system is beyond the scope of the manuscript as it will not provide more information than the ones we will obtain from systematic and extensive co-staining experiments.
Unfortunately, all labs involved are now facing a logistic issue (the animal house at HT is not ready yet, construction works etc) that made importing and setting up of the colony unattainable for the next 6-10months.
If the reviewers and/or the editorial board think this is a major point compromising the entire revision, we kindly ask to contact us again so that we can discuss the issue and arrive to a shared conclusion.

1st Editorial Decision **11th Oct 2022**

Dear Elena,

Thank you for the submission of your peer-reviewed manuscript and your proposed point-by-point response to EMBO reports.

I agree that your study is interesting and potentially a good fit for EMBO reports. If you can address all referee concerns as you propose, we will be happy to re-review your revised manuscript and consider it for publication here.

I would thus like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. 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 major 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 realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (11th Jan 2023). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

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 providing access to data deposited in public databases is missing. If you have not deposited any data, please add a sentence to the data availability section that explains that.
2) Your manuscript contains statistics and error bars based on n=2. Please use scatter blots in these cases. No statistics should be calculated if n=2.

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.

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). See https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf for more info on how to prepare your figures.

3) 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.

4) 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.

5) 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.

6) 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

7) Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database (see https://www.embopress.org/page/journal/14693178/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 & Method (see also https://www.embopress.org/page/journal/14693178/authorguide#datadeposition). Please note that the Data Availability Section

is restricted to new primary data that are part of this study. * Note - All links should resolve to a page where the data can be accessed. *
If your study has not produced novel datasets, please mention this fact in the Data Availability Section.

8) At EMBO Press we ask authors to provide source data for the main and EV figures. Our source data coordinator will contact you to discuss which figure panels we would need source data for and will also provide you with helpful tips on how to upload and organize the files.

9) Our journal also 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 https://www.embopress.org/page/journal/14693178/authorguide#referencesformat

10) Regarding data quantification (see Figure Legends: https://www.embopress.org/page/journal/14693178/authorguide#figureformat)

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.),

- If the data are obtained from n < 3 use scatter blots showing the individual data points.

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.

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

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File (RPF) 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."

I look forward to seeing a revised form of your manuscript when it is ready. Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Best wishes,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Point by point response to Reviewers

We thank the reviewers for their valuable comments regarding our manuscript. We took all comments into account, and we are now presenting the revised version of our manuscript, containing a substantial amount of new data corresponding to all the experiments and analysis requested by the reviewers.

Manuscript number: RC-2022-01528
Corresponding author(s): Elena Taverna and Tanja Vogel

1. General Statements [optional]

We thank the reviewers for the comments and points they raised. We think what we have been asked is a doable task for us. The present revision plan is set to address all points in a manner that we hope the reviewer will find satisfactory.

The title of the manuscript was changed to better fit and describe the data we present and reads as follows:

DOT1L activity affects neural stem cell division mode and differentiation by regulating ASNS expression

2. Description of the planned revisions

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

Reviewer's comment:
The manuscript investigated the role of DOT1L during neurogenesis especially focusing on the earlier commitment from APs. Using tissue culture method with single-cell tracing, they found that the inhibition of DOT1L results in delamination of APs, and promotes neuronal differentiation. Furthermore, using single cell RNA-seq, they seek possible mechanisms and changes in cellular state, and found a new cellular state as a transient state. Among differentially expressed genes, they focused on microcephaly-related genes, and found possible

links between epigenetic changes led by DOT1L inhibition and epigenetic inhibition by PRC2. Based on these findings, they suggested that DOT1L could regulate neural fate commitment through epigenetic regulation. Overall, it is well written and possible links from epigenetic to metabolic regulation are interesting. However, there are several issues across the manuscript.

Response to Reviewer:
We thank the reviewer's 1 for her/his comments and constructive criticism.
We hope the revision plan will address the points raised in a satisfactory manner.

Major issues:

Reviewer's comment:
1) It is not clear whether the degree of H3K79 methylation (or other histones) changes during development, and whether DOT1L is responsible for those changes. It is necessary to show the changes in histone modifications as well as the levels of DOT1L from APs to BPs and neurons, and to what extent the treatment of EPZ change the degree of histone methylation.

Response to Reviewer:
DOT1L protein
We tried several commercially available antibodies, but they do not work in the mouse, even after multiple attempts and optimization. So, unfortunately we will not be able to provide this piece of information
DOT1L mRNA
The data of DOT1L expression at early- (E12), mid- (E14), and late- (E16) neurogenesis using single cell RNA seq from wildtype mouse cerebral cortex is now shown in the Fig. EV1A. DOT1L is transcribed in all cells, but shows quantitative enrichment in some specific cell types. The text was changed accordingly, see page 6:

"DOT1L is expressed in cortical progenitors and neurons throughout early- (E12.5), mid- (E14.5), and late- (E16.5) neurogenesis (Fig. EV1A) [21].

H3K79methylation

After protocol optimization (see Material and Methods), we now provide a staining for H3K79me2 upon treatment in the Fig. EV1B that shows the reduction of the signal upon EPZ inhibition.
Of note, the Con and EPZ treated hemispheres were processed in parallel and acquired at the Zeiss confocal using the same settings (thickness of the optical section, laser power etc.) sequentially in the same day, to be sure the acquisition conditions were comparable for both control and EPZ treatment. In the text we are referring to these additions accordingly (see Results and Material and Methods as well).
The text was changed accordingly, see page 6 and in Figure legends:

"To this end, we treated mouse E14.5 hemispheres with EPZ5676 (EPZ), a widely used inhibitor of DOT1L in cancer research [24-26] and during reprogramming [27]. After 24 h in brain hemisphere rotation (HeRo) culture [28] (Appendix Fig. S1), EPZ reduced H3K79me2 levels (Fig. EV1B)."

***"B)** H3K79me2 stainings of tissue sections upon treatment with DMSO (Con) or EPZ, showing decreased presence of the histone modification upon DOT1L inhibition. Con and EPZ treated hemispheres were processed in parallel, and acquired at the confocal microscope using the same settings sequentially on the same day. Scale bar: 20 µm."*

Reviewer's comment:
Furthermore, the study mainly used pharmacological bath application. DOT1L has anti-mitotic effect, thus it is not clear whether the effect is coming from the inhibition of transmethylation activity.

Response to Reviewer:
We are aware of the potential pitfalls associated with the use of a pharmacological treatment.
We would like here to clarify the reason we decided to use this experimental paradigm.
Our previous work on DOT1L made use of a genetic model (DOT1L conditional KO mouse, Franz et al. 2019).
Although powerful, the genetic model relying on the full ablation of DOT1L did not allow us to understand if the role of DOT1L in brain development is mediated by its scaffolding or enzymatic activity. We wanted to fill this gap in knowledge by focusing specifically on DOT1L's enzymatic activity. This is the reason why we choose to focus on the pharmacological inhibition

with EPZ, whose effect on DOT1L activity has been extensively reported and documented in literature (EPZ is currently in phase clinical 3 studies)
We might have not justified in a convincing way our rationale in the text, to explain the rationale behind the choice of the pharmacological treatment.
We have now added the following text to the manuscript on page 6 to address this concern:

"Previous data, using either genetic ablation in vivo [21] or pharmacological inhibition of DOT1L in vitro [22,23], suggest that DOT1L controls the generation of neurons. However, information is lacking on (i) whether DOT1L exerts its function by its scaffolding or enzymatic activity in vivo, and (ii) which molecular mechanisms are in place. To fill this gap in knowledge, we here specifically focus on DOT1L's enzymatic activity by pharmacologically perturbing DOT1L in the mouse developing brain during mid-neurogenesis."

To address the possible anti-mitotic effect of DOT1L, we experimentally tested the hypothesis by measuring the number of mitotic APs and BPs in control and treated hemispheres by using the standard approach in the field, namely the staining with anti-PHH3 antibody (see also below). We did not observe changes in pHH3 modification, nor in the relative distribution of apical and basal pHH3. We included this information in Fig. EV1E.
The text was changed accordingly, see on page 7:

"To further investigate the effect of DOT1L inhibition on the proliferative or neurogenic potential of cortical progenitors, we first assessed the number and distribution of mitotic, pHH3 positive cells (Fig. EV1E). We observed no difference in the number of pHH3 positive cells, nor in the distribution of pHH3 positive cells between the VZ (apical localisation) and SVZ (basal localisation) (Fig. EV1F). These data suggest that the increased neurogenesis observed upon EPZ treatment was not due to a general anti-mitotic effect of the drug."

Reviewer's comment:
In addition, the study assumed that the effect of EPZ is cell autonomous. However, if EPZ treatment can change the metabolic state in a cell, it would be possible that observed effects was non-cell autonomous. It would be important to address if this effect is coming in a cell-autonomous manner by other means using focal shRNA-KD by IUE.

Response to Reviewer:

We are open on the possibility of a cell autonomous vs non-cell autonomous effect of EPZ. Indeed, we consider both explanations to be potentially valid and it is entirely possible that the effects are non-cell autonomous.

To address the Reviewer's comment, we performed *in utero* electroporation of the DOT1L shRNA followed by immunostainings for TUBB3 and CTIP2.

Our quantification showed an increased neurogenesis (detected as an increase TUBB3 and CTIP2 positive cells) upon KD of DOT1L.

The results of the genetic manipulation thus are in line with what we see when applying the inhibitor, arguing in favor of a cell-autonomous effect of DOT1L.

We modified the text accordingly, and wrote on page 8:

"Finally, we investigated whether we would observe increased neuronal differentiation upon DOT1L knockdown (KD), in a cell-autonomous fashion. To this end we used in utero electroporation and observed that upon DOT1L KD the expression of TUBB3 and CTIP2 increased in GFP-positive, electroporated cells located in the VZ and SVZ (Fig. 2I, J)."

Reviewer's comment:

The possible changes in cell division and differentiation were found by very nice single-cell tracing system. However, changes in division modes occurring in targeted APs such as angles of mitotic division and the expression of mitotic markers were not addressed. This information is critical information to understand mechanisms underlying observed phenotype, delamination, differentiation and fate commitment.

Response to Reviewer:

We thank the reviewer for raising this point. Indeed, in a previous work using DOT1L conditional KO mouse (Franz et al. 2019), we observed a change in the mitotic spindle orientation.

We decided to address this point by quantifying the spindle angle in CON and EPZ-treated cortical hemispheres.

From a practical point of view, we co-stained for DAPI and phalloidin (filamentous actin counterstain) to precisely visualize DNA/chromosomes, the apical surface and the cell contour,

respectively. Of note, we followed the protocol already established in the lab based on published work from the Huttner lab (Taverna et al, 2012; Kosodo et al, 2005).
We then quantified (i) the angle of the cleavage plane in all mitotic phases, and (ii) the partitioning of the apical plasma membrane as an indication of asymmetry of division (Taverna et al, 2012; Kosodo et al, 2005). We did not find any significant differences between control and EPZ. The data strongly suggest that the EPZ effect is not mediated by the previously described effect of DTO1L on mitosis.
We refer to this finding in the Fig. 1G,H. We write in the manuscript on page 9/10:

"DOT1L might affect fate via the reported regulation of mitotic spindle orientation and partitioning of cell biological components [21]. We found no difference in the distribution of mitotic phases upon EPZ treatment, suggesting that EPZ does not cause major changes or alterations in mitosis. We therefore evaluated the cleavage plane angle in mitotic APs and found no changes upon DOT1L inhibition (Fig. 4F, G). Finally, we quantified the partitioning of the apical plasma membrane in mitotic AP, and again found no difference upon DOT1L inhibition (Fig. 4H). Taken together, these data suggest that alteration of the spindle apparatus was not a major driving force of basal fate adoption, but that other mechanism(s) might be at play."

Reviewer's comment:
3) The scRNA-seq analysis indicated interesting results, but was not fully clear to explain the observed results in histology. In fact, in single cell RNA-seq, the author claimed that cells in TTS are increased after EPZ treatment, which are more similar to APs. However, in histological data, they found that EPZ treatment increased neuronal differentiation. These data conflicts, thus I wonder whether "neurons" from histology data are actually neurons? Using several other markers simultaneously, it would be important to check the cellular state in histology upon the inhibition/KD of DOT1L.

Response to Reviewer:
Based on our observations, we indeed found that TTS cells are an intermediate state between APs and neurons in terms of transcriptional profile (for this reason we called this cell cluster transient transcriptional state).
To address the point raised by the reviewer we performed stainings of CTIP2, EOMES and SOX2 in Con and EPZ-treated hemispheres. We observed an increase in CTIP2-expressing

cells (most likely new born neurons) within the VZ, indicating that DOT1L inhibition indeed leads to increased direct neurogenesis and that we observe neurons.
We included this information into the manuscript on page 7/8:

"We further studied the effect of DOT1L inhibition by labelling cycling cells at the end of 24 h HeRo culture with a 1 h EdU pulse, and analysed the fate of EdU-labelled cells by assessing SOX2 and EOMES expression. We observed equal numbers of single SOX2+ or EOMES+ cells, and an increased number of cells that are double positive for SOX2+/EOMES+ upon DOT1L inhibition compared to controls (Fig. 2A, B). Of note, we observed an overall reduced number of cycling, EdU-positive cells. Both SOX2+/EdU+ and EOMES+/EdU+ double positive cells decreased upon DOT1L inhibition compared to controls (Fig. 2A, B), suggesting AP cell cycle exit and acquisition of a differentiative fate, possibly a shorter presence of BPs, or impaired generation of BPs. As SOX2+/EOMES+ cells increased, the latter scenario seemed less likely compared to BPs progressing with differentiation upon DOT1L inhibition.
We further used the Tis21(Btg2)-Gfp mouse to characterise the neurogenic bias upon DOT1L inhibition. Tis21 (Btg2) is a marker expressed by neurogenic APs and therefore the Tis21(Btg2)-Gfp mouse model allows following neurogenesis by monitoring the appearance and localization of GFP (Fig. 2C). Notably, Tis21 is activated in cycling APs undergoing interkinetic nuclear migration and GFP is thus detected within the entire VZ. As a neurogenic marker, Tis21 expression is kept in the neurogenic progeny [32]. Upon DOT1L inhibition we observed an increase in the proportion of cells positive for TIS21-GFP (Fig. 2D), suggesting that more cells adopted a BP and/or neuronal fate [32]. Indication for an increased neurogenic fate was also observed by staining for CTIP2 as a neuronal marker, which indicated arbitrary localisation of these neurons in the VZ upon DOT1L inhibition (Fig. 2E-H)."

Minor issues:

Reviewer's comment:
Figure 1
- It is not clear delaminated cells are APs, BPs or some transient cells (Sox2+ Tubb3+??). It is important to use several cell type-specific and cell cycle markers simultaneously to characterize cell-type specific identity of the analysed cells by staining. These applied to Fig1B,D,E,F,G,as well as Fig2,3.

Response to Reviewer:

We agree with the reviewer. We addressed this point by using a combinatorial staining scheme for several fate markers such as TUBB3, EOMES and SOX2, as suggested by the reviewer.
In the revised manuscript we provide respective experiments showing that DOT1L-inhibition leads to delamination and increased numbers of BPs and neurons.
We show this by stainings for SOX2, EOMES and EdU (Fig. 2A, B), CTIP2 (Fig. 2E-H, I, J) and reference to these findings as outlined in our replies above.

Reviewer's comment:

- Please provide higher magnification images of labelled cells (Fig 1H)

Response to Reviewer:

In the revised manuscript, we will provide higher magnification for the staining.
In the revised Fig 1H (now Fig 2C, right part) we now show higher magnification of the Tis21-GFP labelled cells.

Reviewer's comment:

- Please provide clarification on the criteria of Tis21-GFP+ signal thresholding.

Response to Reviewer:

In the revised manuscript, we provide a clarification on the criteria of Tis21-GFP+ signal thresholding. In the revised Fig. 1H (now Fig. 2C, right part) we now indicate with arrows pointing to the respective cells the signal thresholding for the Tis21-GFP signal.
We are referring to the classification as well in the respective figure legend on page 34/35:

"C) Immunofluorescence staining showing overview of TIS21-GFP-positive (GFP+) cells in VZ of control and EPZ treated sections. Scale bars: 10 µm. Magnifications on the right show exemplarily a TIS21-GFP positive cell (arrow), mild positive cell (arrow with dashed line), and a negative cell (arrowhead)."

Reviewer's comment:

- Splitting the GFP signal between ventricular and abventricular does not convincingly support the "more basal and/or differentiated" states after EPZ treatment.

Response to Reviewer:

We now provide a clarification regarding this point. We have rephrased this statement and do not connect the basal localisation/delamination observation with increased expression Tis21-GFP; rather we treat both observations as independent events.

On page 7 we now write:

"Upon DOT1L inhibition we observed an increase in the proportion of cells positive for TIS21-GFP (Fig. 2D), suggesting that more cells adopted a BP and/or neuronal fate [32]. "

Reviewer's comment:

- Please explain the presence of Tis21-GFP+ cells at the apical VZ.

Response to Reviewer:

Tis21-GFP+ cells at the apical VZ have been extensively reported in the literature, since the first paper by Haubensak et al. regarding the generation of the Tis21-GFP+ line.

In a nutshell, Tis21-GFP+ cells are present throughout the VZ (and also in the apical portion) because neurogenic, Tis21-GFP positive cells are undergoing mitosis at the apical surface. Indeed, the presence of Tis-21 GFP signal have been extensively used by the Huttner lab and collaborators to score apical neurogenic mitosis.

In addition and related to the previous point, Tis21-GFP+ nuclei are going to be present throughout the entire VZ because AP undergo interkinetic nuclear migration,.

In the revised manuscript, we now explain this point and cite additional literature for clarity.

We elaborate on this point on page 7:

"We further used the Tis21(Btg2)-Gfp mouse to characterise the neurogenic bias upon DOT1L inhibition. Tis21 (Btg2) is a marker expressed by neurogenic APs and therefore the Tis21(Btg2)-Gfp mouse model allows following neurogenesis by monitoring the appearance and localization of GFP (Fig. 2C). Notably, Tis21 is activated in cycling APs undergoing interkinetic nuclear migration and GFP is thus detected within the entire VZ. As a neurogenic marker, Tis21 expression is kept in the neurogenic progeny [32]. Upon DOT1L inhibition we observed an increase in the proportion of cells positive for TIS21-GFP (Fig. 2D), suggesting that more cells adopted a BP and/or neuronal fate [32]."

Reviewer's comment:

- Order the legends in same order as the bars.

Response to Reviewer:

Following reviewers' recommendation, in the current revised version the legends and bars are presented in a standardized and clearer way.

Reviewer's comment:

Figure 2

-Fig 2B) The difference between CON and EPZ apical contacts is not clear and does not match with the graph in Fig 2E.

Response to Reviewer:

In the revised manuscript the respective figure is now Fig. 3A.

We extended our explanations of the underlying observation on page 8:

"Having hence successfully implemented AP microinjection, we applied it to study at the single-cell level the effect of DOT1L inhibition on cell morphology, cell identity and lineage progression (Fig. 3A-F). We first analysed the general composition of the progeny of microinjected cells and found that EPZ treatment did not change the proportion of cells found in two-cell clusters as opposed to single-cells (Fig. 3B, C). These data suggest that EPZ does not have nonspecific effects on cell cycle progression per se, nor that it causes mitotic arrest (see also data on pHH3 distribution). The morphological analysis showed that the inhibition of DOT1L resulted in an increase in the number of cells without apical contact, lacking the typical bipolar morphology of APs (Fig. 3B, D) suggesting the generation of a delaminated and more differentiated cell progeny (either BPs or neurons) compared to APs."

In addition, we included in the appendix a new Fig. S3 with panels of Z-stack reconstructions that show apical contacts in control and EPZ condition. We also provide Movies 1-4 for further clarification.

We refer to the novel panel on page 6:

"However, when scoring the distribution of TUBG1- and ARL13B-positive structures specifically in the VZ, we detected a strong increase in abventricular TUBG1- and ARL13B-positive

structures in EPZ treated samples compared to control along with a parallel decrease in ventricular centrosomes and cilia (Fig. 1D-F, Appendix Fig. S2, S3). These data therefore suggest that DOT1L inhibition increases AP delamination, possibly prompting a basal cell fate."

Reviewer's comment:
-Supp Fig 2 - are these injected slices cultured in control conditions? Please include this in the text and figure/figure legend

Response to Reviewer:
We added the respective information into the text and the corresponding figure legend.
On page 8 we write:

"APs were microinjected with Dextran-Alexa555 in organotypic slices from E14.5 wildtype mouse telencephalon (see scheme in Appendix Fig. S1). The slices were kept in culture for various time windows to reconstruct and study the AP progeny. Cell identity was defined by combining several criteria, such as cell morphology, location and marker expression [29,30,33-35]. 3 h after microinjection the progeny of microinjected APs resided almost exclusively in the VZ and had contact with the ventricle (Appendix Fig. S4A). Instead, 24 h after microinjection the progeny of microinjected APs (i) located not only to the VZ, but also to the SVZ and CP, and (ii) expressed the neuronal marker TUBB3 (Appendix Fig. S4B, panels on the right)."

And the legend to the Appendix Fig. 4:

"Appendix Fig. S4. Additional information on microinjection and lineage tracing experimental procedure. A) Overview of microinjected tissue section in wildtype condition without treatment showing Dx-A555 labelled cells after 3 h slice culture. B) Microinjected cells and progeny expressing EOMES and/or TUBB3 after 24 h slice culture in wildtype condition without treatment."

Reviewer's comment:
Fig 2C) The EPZ-treated DxA555+ cells exhibit morphological change of cell shape. Is this phenotype? please comment on the image shown for EPZ treatment panel.

Response to Reviewer:

We thank the reviewer for raising this very interesting point. We think that the change in morphology might be a consequence of delamination and/or of cell fate, as the two processes are strictly intermingled even in physiological conditions. In the revised manuscript, to address the reviewer point, we changed the sentence on page 8/9 and explicitly mentioned APs morphology.
The sentence now reads as follows:

"The morphological analysis showed that the inhibition of DOT1L resulted in an increase in the number of cells without apical contact, lacking the typical bipolar morphology of APs (Fig. 3B, D) suggesting the generation of a delaminated and more differentiated cell progeny (either BPs or neurons) compared to APs."

Reviewer's comment:
Fig 2F - 2G) Data presented on EOMES+ and TUBB3+ % are counterintuitive. The authors claimed that TUBB3+ cells are increased and neuronal differentiation is promoted. However, no changes in EOMES+ are observed. What is the explanation? Did the author check the double positive cells? These could be TSS cells?

Response to Reviewer:
We thank the reviewer for raising this point.
As suggested by the reviewer, we suspect that the counterintuitive data might be due to TTS cells, which based on our scRNAseq data are expressing at the same time several cell type specific markers. It is possible that, since the treatment with EPZ is 24h long, cells (like the TTS cluster) have no time to eliminate the EOMES protein. If that were to be the case, then we would expect to still detect (as we indeed do) EOMES immunoreactivity.
To address this point, as suggested, we analyzed the scRNA-seq data for the co-expression of Eomes and Tubb3. The strongest co-expression is observed in the neurons cluster (Fig. EV4E). The cells in TTS do not show a particular co-expression, but the cluster contains cells positive for either Eomes or Tubb3.
Seemingly, the increased number of TTS cells contributes to increased Eomes positive cells alongside differentiated neurons that retain to some extent the transcription of Eomes and co-express Tubb3. This might indicate that in the time window of observation (24h), cells are also not eliminating EOMES protein expression completely upon DOT1L inhibition, which possibly accounts for comparable numbers to the DMSO treated condition (control), despite of the

expectation that EOMES-expressing should decrease upon increased neurogenesis. This finding is supported by the pseudotime trajectory analyses using Slingshot indicating that both APs and BPs contribute to the TTS (Fig. EV4C). We took this finding into account and rephrased the summary regarding the histological findings accordingly.
We change the text accordingly, see page 12:

"To address the question of whether the TTS expressed differentiative marker genes, we plotted the co-expression of Eomes and Tubb3 in the electroporated data set, which showed that very few cells classified as TTS co-expressed both markers (Fig. EV4E). Instead, the TTS cluster contained cells expressing either Eomes or Tubb3 at different levels, and cells negative for both markers."

In a separate set of immunofluorescence experiments, we checked for Sox2, EOMES and CTIP2 double positive cells in hemispheres treated with EPZ drug (Fig 1E-H). We observed no difference in the total number of Sox2 positive and EOMES positive cells. Of note, in the EPZ treated samples, we do observed an increase in the number of Sox2 and EOMES double positive cells, suggesting an increase of a state of transition between the AP and BP fate. This data also suggest a general increase in the tendency of APs to a more basal fate, in agreement with the described increase in the TUBB3 positive cells.
We think this might possibly be the elusive TTS state revealed by scRNAseq data - although we are aware of the challenge of detecting a cell state, by definition transient and limited to only a subpopulation of cells, using immunofluorescence (for this precise reason, and to avoid any overinterpretation, we used a cautious phrasing).
We summarised our findings on pages 8 and 9:

"Our data combining cell biological enquiry, analysis of cell distribution and fate specification via marker expression, suggest that upon DOT1L inhibition the AP progeny is committed to a delaminated, more basal and differentiated fate (possibly BP/neuronal).

"Taken together, these data suggest that the inhibition of DOT1L might favour APs to acquire a differentiative fate that results in the generation of increased numbers of neurons."

Reviewer's comment:
Figure 2 and Figure 3) the number of pairs analyzed for EPZ is twice as that of Con for

comparison of the parameters taken into account. Please include n of each graph in the figure legend of the specific panel if not the same for all panels in that figure (i.e. for figure 3)

Response to Reviewer:

According to the reviewer's suggestion we included all quantitative information in the revised legend to Fig. 4 on page 36 where in the revised manuscript we display the respective data. The text reads as follow:

"For quantifications the following number of cells were scored: TUBB3 - 52 daughter cell pairs in total, Con: 19, EPZ: 33 pairs; EOMES - 43 daughter cell pairs in total, Con: 16, EPZ: 27 pairs; TIS21-GFP - 64 daughter cell pairs in total, Con: 35, EPZ: 29 pairs; apical contact - 56 daughter cell pairs in total, Con: 19, EPZ: 37 pairs. Quantifications are from three independent experiments (n=3), 260 cells in total (Con: 86 cells, EPZ: 174 cells)."

Reviewer's comment:

Figure 3)

The data indicated that the number of daughter cell pairs in EPZ samples is almost double than Control. Is this the phenotype? More numbers of daughter cells in EPZ treated samples were observed from the same number of injections? or the number of injected cells were different?

Response to Reviewer:

Due to technical reasons, we performed a higher number of injections in EPZ-treated slices. We think this is the main reason behind the difference in number.

If the reason were to be biological, one would expect to see the same trend in IUE experiments, but this is actually not the case. This does suggest/corroborate the idea that the reason behind the difference in microinjection experiments is mainly technical.

We now mention the source of this difference being in the Material and Methods section.

On page 21 now the sentence reads as follows:

"Automated microinjection into single APs in tissue was used for scRNA-seq experiments, where a higher throughput was needed (note that for the slices we planned to treat with EPZ, we selected slices that contained on average a higher number of microinjected cells)."

Reviewer's comment: Figure 4)

- Please clarify if the single cell transcriptomic analysis has been performed only once, and if yes, how statistical testing to compare the cell proportion is carried out with only one batch. Fig 4G)

Response to Reviewer:

As for the scRNAseq on microinjected cells:
the scRNA-seq analysis was done once using cells pooled from 3 different microinjection experiments performed in 3 different days.
As for the scRNAseq on IUE cells:
The scRNA-seq analysis was done once using cells pooled from 2-3 different IUE experiments performed in 3 different days.
For all scRNAseq experiments the statistical testing is achieved by intrasample comparisons according to established bioinformatics pipelines.
We now provide this information in the Methods section on page 23:

"For scRNA-seq of microinjected cells we pooled FACS-isolated cells from three different microinjection experiments, which were performed on three different days. ScRNA-seq of electroporated cells was done once using cells pooled from 2-3 different electroporation experiments, which were performed on three different days. For all scRNA-seq experiments the statistical testing was performed using intra-sample comparisons, according to established bioinformatics pipelines (see below)."

Reviewer's comment:

Figure 4 and 5)
- Figures are not supportive of the statement regarding APs' neurogenic potential upon DOT1L inhibition. TSS transcriptomic profile resembles more progenitors than neurons. Please comment on TSS neurogenic capacity taking into account the provided GO and RNAseq.

Response to Reviewer:

We thank Reviewer 1 for raising this point. It is indeed true that although TTS have a mixed signature, they do resemble more AP than neurons (as indicated in the Fig. S5B, C). We interpreted this finding the mean that these cells are transient and therefore still maintain some AP features. Interestingly, TTS downregulate cell division markers, suggesting a restriction of proliferative potential, as one would expect for cells with an increased neurogenic potential.

Following the reviewer's comment, we added new data and characterisation of TTS in the revised manuscript (Fig. EV2, 3, 4). This point is also addressed in the respective comment below in regard to the TTS characterization, we are giving the detailed information addressing this comment below.

Reviewer's comment:

- Please provide GO analysis for APs and BPs.

Response to Reviewer:

Following the reviewer's suggestion, we now incorporate a more careful and in-depth analysis in the revised version of the manuscript, by providing GO term analysis in the Fig. EV3A-F (see also Data set EV1).

We refer to them on page 11 and 13:

"Further, GO enrichment analysis (electroporation dataset) showed that genes up-regulated in the TTS compared to APs or BPs were enriched in terms associated with apical hallmarks (Fig. EV3A, B). Genes with decreased expression in the TTS cluster compared to APs or BPs were enriched in GO terms related to cell division (spindle and centrosome organisation, midbody, kinetochore and chromatin condensation, cyclin-dependent protein kinases) and early neuronal differentiation (distal axon) (Fig. EV3A, B). Comparing the GO terms of TTS and neurons showed a decrease of genes associated with neuronal differentiation (Fig. EV3C). Together, this transcriptional signature of TTS suggests that this cell state relates best to APs with reduced proliferation capacity."

"Intriguingly, analysis of GO terms that were enriched in APs and BPs revealed that the DEGs upon DOT1L inhibition included metabolism and oxidative phosphorylation (OxPhos), a hallmark of neuronal differentiation, suggesting thus a potential alternative mechanism compared to spindle orientation (Fig. EV3D, E). The DEGs in the TTS also enriched for GO terms pointing towards metabolic changes, but also towards a more differentiative state upon DOT1L inhibition compared to control (Fig. EV3F, Appendix Fig. S6)."

Reviewer's comment:

- Reconstruct figure 5A by listing genes in the same order in both Con and EPZ and prioritize EPZ-Con differences instead of cell-cell differences.

Response to Reviewer:

We revised Figure 5A based on the reviewer's comment. SCENIC, the tool we used to infer TF activities, is based on the transcriptional profile in the cells. Control and treated cells are split prior to this analysis to capture data specific TF activities. This procedure bears two important consequences:

First, the same TFs are not necessarily captured in control and treated conditions (see below). Second, the TFs captured are clustered to show their relationship to the other TFs in the respective data set, i.e. treatment condition. This is indicated by the cluster information on the left of the figure. Reordering the TFs by hand consequently results in a loss of this information. To follow the suggestion of the reviewer, we nevertheless provide a TF centered comparison in the Fig. EV5A, in a similar style to the Fig. 6E, which highlighted EZH2 in the two conditions. We show all TF shared between the two conditions, DMSO and EPZ.

We write on page 13:

"The analysis of changes in the TF network activity in response to DOT1L inhibition, in a cell-type resolved manner, was done using the SCENIC package [51], that revealed a set of key transcriptional regulators with treatment and cell-type specific activity changes (Fig. 6A, Fig. EV5A). Atf4, Cepbg, Ets1, 2, Ezh2, JunB, Pou3f1, Pou32f2, Rad21, Sox2, 4, 9, 11, 21, Tcf7l1 and Tcf4, which were present in both treatment conditions, changed activity upon DOT1L inhibition in APs, BPs or TTS."

Reviewer's comment:

Moreover, the presented genes in the heatmap is not the same in two conditions (i.e. NEUROG1 is present in EPZ but absent in Con). Please justify.

Response to Reviewer:

This observation is based on different activities of transcription factor networks in the control and EPZ condition. They are not necessarily supposed to be the same as the cell states are altered and different TF are expressed and active upon the treatment in the diverse cell types. In regard to the differences of TF that are captured in the one or the other condition, this is an expected outcome, as the epigenetic landscape changes and is followed by diverse activations/inactivations of genes. These can be either TF, or chromatin remodelers that allow

TF to bind or to fall off from the chromatin. Therefore, in different treatment conditions we expect differences in the TF network activities.
We refer to the specific alterations between the treatment conditions in the text on page 13 with the text outlined in our comment above.

Reviewer's comment:
Fig 5D)
- Please explain why binding of EZH2 on the promoter of Asns is strongly reduced in comparison to a mild significant reduction of H3K79me/H3K27me3 in EPZ compared to Control.

Response to Reviewer:
We can envisage several explanations
First, the simpler explanation could be that the variation is due to batch effects.
Second, the acute reduction of EZH2 might not be directly accompanied by a reduced histone mark, which is reduced either by cell division or by demethylases. The two processes of getting rid of the mark might be slower than the reduction of EZH2 presence at the respective site.
Following to the reviewer's comment we included the explanation about different kinetics of EZH2 recruitment and H3K27me3 erasion in the discussion on page 17:

"Notably, the acute and strong reduction of EZH2 might not be directly followed by a comparable reduction in H3K27me3, as shown in Fig. 6F. H3K27me3 can be erased either by cell division or by demethylases, which are two processes that might have slower kinetics than the recruitment of EZH2 at the respective genomic loci."

Reviewer's comment:
Also is the changed directly medicated by DOT1L?
Please test whether DOT1L can bind the promoter of Asns.

Response to Reviewer:
To address this relevant issue, during the revision phase we first tried the following protocol:

(i) electroporate a tagged version of DOT1L into ESCs or NPCs
(ii) select ESCs and differentiate them into NPC_48h.
(iii) treat NPC with DMSO (Con) or EPZ

(iv) harvest CON and EPZ-treated NPC

(v) perform ChIP-qPCR DOT1L at the Asns promoter

Our attempts to obtain a sufficient number of cells expressing tagged DOT1L after electroporation of either mESCs or NPCs were unfortunately unsuccessful.

We were therefore not able to conduct the experiment as envisioned in our revision plan.

As an alternative, we established the ChIP using DOT1L antibody directly from *in vivo* tissue, as this strategy is also more directly comparable to the rest of the work performed in our study.

The enrichment of precipitated chromatin at the *Asns* TSS suggests that DOT1L is present and binds to the promoter region, as seen by the ChIP enrichment compared to IgG.

As DOT1L is the only confirmed histone methyltransferase mediating H3K79 methylation, presence of the mark is a valid, though indirect, readout for presence of DOT1L as well.

We now provide the respective figure showing indeed enrichment of DOT1L at the Asns promoter region in the Fig. EV5D. Accounting for the finding that DOT1L binds the *Asns* promoter in vivo, we also modified the Fig. 6D and included the H3K79me2 pattern of cortical tissue at E12.5 and E14.5 in the ChIP-seq profiles.

We modified the text accordingly on page 14:

"H3K79me2 was also marking Asns in cortical tissue (Fig. 6D), and ChIP-qPCR using cortical tissue suggested that DOT1L was present at the Asns promoter (Fig. EV5D)."

Reviewer's comment:

Please provide the expression patterns of DOT1L and Asns during neuronal differentiation.

Response to Reviewer:

As for DOT1L

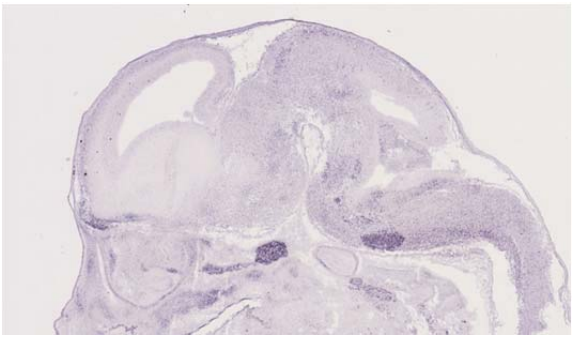
We are providing the expression pattern during neurogenesis for Dot1l in the Fig. EV1A and added the relevant citation showing expression of Dot1l using in situ hybridisation. We tried to optimize immunofluorescence staining, but (despite several attempts) the antibody against DOT1L does not work in our hands.

On page 6 we write:

"DOT1L is expressed in cortical progenitors and neurons throughout early- (E12.5), mid- (E14.5), and late- (E16.5) neurogenesis (Fig. EV1A) [21]."

As for ASNS

We provide the in situ hybridisation for Asns at E14.5 (Gene Paint database, see below) and expression levels of Asns transcripts in early-, mid- and late-neurogenesis as revealed by scRNA-seq in the Fig. EV5B, C.



In addition, we optimized the staining protocol using the ASNS antibody, and we now show the increased ASNS protein expression upon DOT1L inhibition at E14.5 in the Fig. 6C.
We added the text on page 13/14:

"In this set of target genes, the increased expression of Asns was our top candidate as misexpression of Asns is not only involved in leukaemia [52] (for which deregulated functions of DOT1L have been described [53]), but its mutation also causes microcephaly [54], and it is expressed during cortical development (Fig. EV5B, C). However, DOT1L is generally considered to favour transcription, thus increased expression of ASNS upon DOT1L inhibition, as confirmed on the protein level (Fig. 6C), is seemingly counter-intuitive."

Other General comments:

Reviewer's comment:
Please Indicate VZ, SVZ and CP on the side of the pictures/ with dot lines in the pictures both for primary figures and supplementary.

Response to Reviewer:
We are now provide our figures with the respective labels throughout the manuscript.

Reviewer's comment:

- The Results and figures sometimes do not support the statement made by the authors

Response to Reviewer:

We carefully checked and revised the text and eliminated any possible overinterpretation of the data. We hope the reviewer will find the phrasing satisfactory.

Reviewer's comment:

Schemes are not informative/explanatory enough, i.e. time windows of treatment and sample collection, culture conditions details.

Response to Reviewee:

We now provide in Appendix Fig. S1 a compilation of all methods in much more detail.

Reviewer's comment:

A more extensive characterization of TTS cells in terms of differentiation progression and integration would be enlightening

Response to Reviewer:

We thank the reviewer for pointing her/his attention on the TTS cell cluster.

Although the TTS population is intriguing, our ability to carry out an in-depth analysis of this cell state is limited. This is because of two reasons. One is the lack of a specific marker gene for TTS, the other is the relatively small size of the TTS subpopulation. Bearing in mind these limitations, we develop the following experimental and computational plan to meet the reviewer's comment and expectations.

We provide a more extensive characterisation of the TTS cells by including single molecule FISH expression data for two marker genes and by additional bioinformatics analysis. TTS cells do not express single marker genes that would allow an unequivocal identification using immunofluorescence methods.

We used smFISH to visualize (i) Ofd1, a gene with enriched expression in TTS identified from microinjected cells scRNAseq data, and (ii) Nr2f1, a gene with enriched expression in TTS identified from electroporated cells scRNAseq data. We highlight cells co-expressing these markers in the Fig. EV2E, which might be TTS cells. However, we cannot judge that these cells are exclusively TTS, as the markers are not exclusive for this cell state. In addition, since TTS

seems to be a cell state and not a cell type, its transient nature might represent a challenge for detection using standard immunofluorescence data.
In addition, we now provide in the revised manuscript GO terms analysis for APs, BPs, TTS upon DMSO and EPZ treatment, as well as for TTS vs APs, TTS vs BP and TTS vs Neurons (Fig. EV3A-F, see also Data set EV1), predicted lineage trajectory using scvelo (Fig. EV4B), using Slingshot (Fig. EV4C), and using Monocle (Fig. EV4D). We also analysed the co-expression of Eomes and Tubb3 in the TTS (Fig. EV4E). Taken all results of these analyses together, we conclude that (i) APs are preceding the TTS, (ii) that BPs give rise to this state esp upon DOT1L inhibition and (iii) that the TTS is not co-expressing Eomes and Tubb3 to a major extent, but that TTS contained cells that express Eomes or Tubb3, or are negative for both.
In the revised manuscript we included the extensive characterisation of the TTS on pages 11 and 12:

"While the TTS comprised a minor fraction in control conditions, its proportion increased upon EPZ treatment, together with a decrease in the proportion of APs (Fig. 5D, G). We exemplarily conducted single molecule FISH experiments using probes against Ofd1 and Nr2f1, which showed an increased expression upon DOT1L inhibition compared to controls (Fig. EV2E). To further characterise the TTS, we plotted the expression levels of selected cell type/state markers in APs, TTS, BPs and neurons (Fig. EV4A). The best fitting overlap was again between APs and TTS, but differences were clearly observable. The data so far suggested that TTS's transcriptional makeup described above had hallmarks of APs, but had at the same time a decreased proliferative capacity (for example reduced expression of Cdk-complex members [46] including Ccna2, Cdk1, Ccnb1, Cdk6, Cks2, Cks1b, Ccnb2, Ccnd2, Pcna, Ccnd1, Ccng2, Appendix Table 2). As the number of TTS increased upon DOT1L inhibition at the expense of APs, the scRNA-seq data reflected at the transcriptional level our data on single-cell reconstruction of lineage progression and a decrease in proliferative capacity observed in the EdU pulse experiment (Fig. 2B).
We next addressed whether the scRNA-seq data would also support a general increase in the differentiative fate of APs upon DOT1L inhibition. To this end we aimed at inferring lineage trajectories from the electroporated scRNA-seq data. We could not use scVelo, an algorithm that predicts trajectories on the basis of the ratio of spliced and unspliced transcripts [47], as in our dataset the proportions between spliced/unspliced transcripts were equally distributed, and the inferred lineage trajectory were against the known biological relation, i.e. neurons projecting towards progenitors (Fig. EV4B). Instead we used two cluster-based prediction tools, Slingshot

[48] and Monocle [49]. In the control condition, both algorithms revealed that APs have a higher probability to generate the TTS, which can resolve in BPs and/or neurons (Fig. EV4C, D). Upon DOT1L inhibition, Slingshot predicted that both APs and BPs differentiate towards the TTS, which gives rise to neurons, in accordance with our extensive histological marker expression analysis shown before. The lineage trajectories were less clear using Monocle, predicting the TTS followed both APs and BPs, but failing to connect the TTS directly to neurons (Fig. EV4C, D). To address the question of whether the TTS expressed differentiative marker genes, we plotted the co-expression of Eomes and Tubb3 in the electroporated data set, which showed that very few cells classified as TTS co-expressed both markers (Fig. EV4E). Instead, the TTS cluster contained cells expressing either Eomes or Tubb3 at different levels, and cells negative for both markers. This observation suggested that the TTS reflects an uncommitted cell state that may directly follow after the end of mitosis when cell-specific transcription is reinitiated [50]."

Reviewer's comment:

- Picture quality can be improved, provide high magnification images.

Response to Reviewer:

We have included higher magnification images, and novel images to improve on the figure quality.

Reviewer #1 (Significance (Required)):

Reviewer's comment:

The study could be important for the specific field in neural development.
It aims to understand mutations in respective genes and brain malformation.
If the link between epigenetic and metabolic changes is clearly shown, it will be interesting.
However, the current manuscript is still rather descriptive, and clear mechanistic insights were not provided. The study have potentials and additional data will strength the value of study.

Response to Reviewer:

We thank the reviewer for the constructive comments and criticism.
During the revision we addressed the direct impact of DOT1L and H3K79me2 on the *Asns* gene locus (see the rationale of the experimental strategy also in the revision plan above).

By doing so, we hope we have strengthened further the mechanistic link between epigenetics and altered metabolism.

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

Reviewer's comment:

Appiah et al. present a concise manuscript that provides details and possible mechanisms of their previous work (Franz et al., 2019; Ferrari et al., 2020). The study uses diverse lines of investigation to arrive at most conclusions. However, as interesting as the data is, we find that at the present state, it is not sufficient to prove that, indeed, the asparagine metabolism is regulated by DOTL1/PRC2 crosstalk.

The neurogenic shift presented in the first part of the paper is not comprehensive and, therefore, not very convincing. The quality of images provided in the main and supplementary data is less than ideal.

Additional data analysis and interpretation of the scRNA seq data may be needed.

The authors finally conclude with rescue experiments done in culture and in-vivo, which we believe is the stand-out part of this study.

Overall the manuscript has some interesting observations that are often over-interpreted with less supporting data. The manuscript reads well but requires additional data and changes in the claims/interpretation to be suited for publication.

Response to Reviewer:

We thank the reviewer for the constructive comments and criticism. Based on her/his comments, we designed an experimental plan that we hope address comments and concerns in a satisfactory manner.

Reviewer's comment:

1) Abstract:

Is this statement correct: "DOT1L inhibition led to increased neurogenesis driven by a shift from asymmetric self-renewing to symmetric neurogenic divisions of APs. AP undergoes symmetric division for self-renewal and asymmetric neurogenic divisions.

Response to Reviewer:

Based on the current literature (cit. Huttner and Kriegstein), AP undergo:

- symmetric division for proliferative division *at early stages of neurogenesis*
- asymmetric self-renewing division, generating an AP and a BP *at mid neurogenesis*. This division is also described as neurogenic, as it produces a BP, that is a step further than AP in term of neurogenic potential.
- symmetric consumptive division *at late neurogenesis*

To avoid any possible confusion, we changed the abstract taking this reviewer's concern into account.
The text on page 3 now reads as follows:

"Combining lineage tracing with single-cell RNA sequencing of clonally related cells, we show at the cellular level that DOT1L inhibition led to increased neurogenesis driven by a shift of APs from asymmetric self-renewing (generating one AP and one basal progenitor) to symmetric consumptive divisions (generating two neurons)."

Reviewer's comment:
All the data is based on treatments with EPZ (DOTL1 inhibitor), yet no information is shown to support its targeted activity in this system. A proof of principle in the chosen experimental system is missing; for instance, examining the activity or protein level of DOTL1 and decreased methylation of the target(s) is essential.

Response to Reviewer:
We agree with the points raised by the referee and we plan to it by assessing the methylation state upon EPZ treatment (see below).
In general, EPZ is a well characterized drug, that has been used previously in our lab and by others as well. As for the Vogel lab, the information regarding EPZ , its activity and efficiency in inhibiting DOT1L towards H3K79me2 was shown in Franz et al. 2019, Supplementary Fig. S6 D, E.
In the present manuscript, an additional confirmation that EPZ targets DOT1L in regard to its H3K79me2 activity is shown in Fig. 5D. We now refer to this information more explicitly in a revised manuscript.
In addition, ee performed immunostainings for H3K79me2 in CON and EPZ (please refer to the same comment of reviewer 1).
We included this information in the Fig. EV1B and inserted the following text on page 6:

"To this end, we treated mouse E14.5 hemispheres with EPZ5676 (EPZ), a widely used inhibitor of DOT1L in cancer research [24-26] and during reprogramming [27]. After 24 h in brain hemisphere rotation (HeRo) culture [28] (Appendix Fig. S1), EPZ reduced H3K79me2 levels (Fig. EV1B)."

Reviewer's comment:
2) Figure 1: The scoring of centrosomes and cilia is insufficient to conclude delamination and increase in basal fates. The effect could be on ciliogenesis or centrosome tethering to the apical end-feet of the AP, and other possible explanations for this observation also exist. The images are too small; larger images or graphic representations could be helpful in addition to the data.

Response to Reviewer:
We agree with the reviewer's comment. We in fact did not want to claim that the change in centrosome location *demonstrates* delamination, but only that it *suggests* delamination.
To avoid any misunderstanding regarding our interpretation of that data, we re-phrased in a more cautious way the text referring to Figure 1 to highlight that the data *only suggest* delamination.
We revised the manuscript and toned down our finding in regard to Fig. 1. on page 6:

"These data therefore suggest that DOT1L inhibition increases AP delamination, possibly prompting a basal cell fate."

We also explained in the material and methods our approach in the context of the present literature. Indeed, the relocation of the centrosomes has been extensively used as a proxy for delamination by several labs working on the cell biology of neurogenesis, such Huttner and Gotz labs. We will therefore refer to this work more clearly in the revised manuscript.
Based on that, we added information to the material and methods section on page 27:

"APs mitosis. The orientation of the cleavage plane in mitotic APs relative to the ventricular surface and the partitioning of the apical plasma membrane were determined in optical sections of mitotic APs cells stained for DNA and phalloidin (to visualise the membrane and cell's contour), following the protocol developed by Kosodo [74]. "

And in the Appendix Fig. S2, S3 we provide better images of cell delamination, backup by several movies of the resolved z-stacks, and refer to the figures on page 6:

“However, when scoring the distribution of TUBG1- and ARL13B-positive structures specifically in the VZ, we detected a strong increase in abventricular TUBG1- and ARL13B-positive structures in EPZ treated samples compared to control along with a parallel decrease in ventricular centrosomes and cilia (Fig. 1D-F, Appendix Fig. S2, S3). These data therefore suggest that DOT1L inhibition increases AP delamination, possibly prompting a basal cell fate.”

Reviewer’s comment:
To make a statement regarding delamination, I would like to see either the dynamics of delamination (organotypic slices images), staining with BP markers, or morphological changes of AP (staining that will reveal loss of adherence) or comparable data to support the observation. In my opinion Supp. Figure 1 is insufficient; the single image is not convincing; I would like to see 3D reconstruction and better-quality images.

Response to Reviewer:
We now provide additional images and co-stainings; please refer to the comment below for further details. We think it is beyond the scope of the manuscript embarking in live imaging as we are not studying the dynamics of delamination *per se*.

Reviewer’s comment:
Tis21 data (1H), again of low quality, is only a single piece of evidence and the conclusion "suggesting that the acquisition of a basal fate was paralleled by a switch to neurogenesis" is premature. I think other cell cycle exit reporters, Fucci markers, pHis, BrdU, NeuroD, or Tbr2 reporters (Li et al., 2020, (Haydar and Sestan labs)) to name a few, are necessary to establish the conclusions. The authors should show other markers such as PAX6, EOMES, or other upper-layer markers upon cell cycle exit in the SVZ/CP. These additional experiments will assist in cell fate analysis.

Response to Reviewer:
We completely understand the points raised by the reviewer, and we now provide in the new Fig. 2A,B stainings and quantifications of EdU, SOX2, EOMES, in Fig. EV1E pHH3 stainings,

and in Fig. 2E-J stainings and quantifications with CTIP2 to undermine our claim of increased differentiation and reduced proliferation upon DOT1L inhibition.
We rephrased the manuscript accordingly on pages 7 and 8:

"To further investigate the effect of DOT1L inhibition on the proliferative or neurogenic potential of cortical progenitors, we first assessed the number and distribution of mitotic, pHH3 positive cells (Fig. EV1E). We observed no difference in the number of pHH3 positive cells, nor in the distribution of pHH3 positive cells between the VZ (apical localisation) and SVZ (basal localisation) (Fig. EV1F). These data suggest that the increased neurogenesis observed upon EPZ treatment was not due to a general anti-mitotic effect of the drug.
We further studied the effect of DOT1L inhibition by labelling cycling cells at the end of 24 h HeRo culture with a 1 h EdU pulse, and analysed the fate of EdU-labelled cells by assessing SOX2 and EOMES expression. We observed equal numbers of single SOX2+ or EOMES+ cells, and an increased number of cells that are double positive for SOX2+/EOMES+ upon DOT1L inhibition compared to controls (Fig. 2A, B). Of note, we observed an overall reduced number of cycling, EdU-positive cells. Both SOX2+/EdU+ and EOMES+/EdU+ double positive cells decreased upon DOT1L inhibition compared to controls (Fig. 2A, B), suggesting AP cell cycle exit and acquisition of a differentiative fate, possibly a shorter presence of BPs, or impaired generation of BPs. As SOX2+/EOMES+ cells increased, the latter scenario seemed less likely compared to BPs progressing with differentiation upon DOT1L inhibition."

"Indication for an increased neurogenic fate was also observed by staining for CTIP2 as a neuronal marker, which indicated arbitrary localisation of these neurons in the VZ upon DOT1L inhibition (Fig. 2E-H). Finally, we investigated whether we would observe increased neuronal differentiation upon DOT1L knockdown (KD), in a cell-autonomous fashion. To this end we used in utero electroporation and observed that upon DOT1L KD the expression of TUBB3 and CTIP2 increased in GFP-positive, electroporated cells located in the VZ and SVZ (Fig. 2I, J)."

We think establishing the Fucci or EOMES mouse system is beyond the scope of the manuscript. In addition, given the present setting of all labs involved (we relocate in a new institute and our animal facility is not yet open, and we can use only wt animal in a separate institute), it would be logistically unattainable (see also comments in section 4.).
We think and hope the co-staining scheme is informative enough to satisfactory address the concerns raised by the reviewer.

Reviewer's comment:

2) Figure 2: The microinjection experiments are elegant; the images, however, do not complement the experiment. The images of the microinjected cells seem not to be reconstructed from z-stacked optical slices, so often, processes are not continuous (panel B, for example); therefore, it is not clear if an apical process is indeed missing or just not seen.

Response to Reviewer:

The mentioned images are reconstructed from continuous Z-stacks, as we always do given the type of data. We have included the Appendix Fig. S3 showing the continuous z-stacks, and we provide in the Appendix movies that allow browsing through the z-stacks to demonstrate the delamination phenotype upon DOT1L inhibition.

Reviewer's comment:

The data analysis should include other parameters; BrdU staining could have given information on cell cycle exit, PAX6, SOX2, and EOMES on the location of the cells in the VZ/sVZ. The quality of images showing EOMES and TUBB3 staining is so low that it makes the reader doubt the validity of the quantifications.

"Taken together, these data suggest that the inhibition of DOT1L might favor the acquisition of a neuronal over BP cell fate" This interpretation should be subjected to more investigations. It is possible that this treatment just accelerates the AP-> BP -> Neuronal fate.

The author's claim needs to be backed by additional experiments or be changed.

Response to Reviewer:

To address this valid concern of our reviewer, in the revised manuscript we provide stainings for SOX2, EOMES, and EdU as well as CTIP2. We provide evidence that we observe increased neurogenesis instead of accelerated neurogenesis.

On page 7 we write:

"To further investigate the effect of DOT1L inhibition on the proliferative or neurogenic potential of cortical progenitors, we first assessed the number and distribution of mitotic, pHH3 positive cells (Fig. EV1E). We observed no difference in the number of pHH3 positive cells, nor in the distribution of pHH3 positive cells between the VZ (apical localisation) and SVZ (basal

localisation) (Fig. EV1F). These data suggest that the increased neurogenesis observed upon EPZ treatment was not due to a general anti-mitotic effect of the drug.
We further studied the effect of DOT1L inhibition by labelling cycling cells at the end of 24 h HeRo culture with a 1 h EdU pulse, and analysed the fate of EdU-labelled cells by assessing SOX2 and EOMES expression. We observed equal numbers of single SOX2+ or EOMES+ cells, and an increased number of cells that are double positive for SOX2+/EOMES+ upon DOT1L inhibition compared to controls (Fig. 2A, B). Of note, we observed an overall reduced number of cycling, EdU-positive cells. Both SOX2+/EdU+ and EOMES+/EdU+ double positive cells decreased upon DOT1L inhibition compared to controls (Fig. 2A, B), suggesting AP cell cycle exit and acquisition of a differentiative fate, possibly a shorter presence of BPs, or impaired generation of BPs. As SOX2+/EOMES+ cells increased, the latter scenario seemed less likely compared to BPs progressing with differentiation upon DOT1L inhibition."

Reviewer's comment:
3) Figure 3: The experiment concept and its performance are impressive, yet the data is insufficient. The images in A that are supposed to be representative show two cells; their location is not clear, and the expression of GFP is not clear; in fact, both pairs seem to be GFP negative (not clear what is the threshold for background). Staining with anti-GFP and a second method to follow neurogenesis is necessary.

Response to Reviewer:
We thank the Reviewer for her/his general comment on our work.
In line with the reviewer comments in the need of deepening our analysis, in the revised manuscript we provide staining for CTIP2 (Fig. 2E-J) for CON and EPZ treated brain hemispheres. With the intent of providing a deeper and more specific analysis of the phenotype we described, we also knocked down DOT1L via shRNA in utero electroporation and we provide the staining for CTIP2 for DOT1L KD as well.
We write on page 7,8:

"Indication for an increased neurogenic fate was also observed by staining for CTIP2 as a neuronal marker, which indicated arbitrary localisation of these neurons in the VZ upon DOT1L inhibition (Fig. 2E-H). Finally, we investigated whether we would observe increased neuronal differentiation upon DOT1L knockdown (KD), in a cell-autonomous fashion. To this end we used

in utero electroporation and observed that upon DOT1L KD the expression of TUBB3 and CTIP2 increased in GFP-positive, electroporated cells located in the VZ and SVZ (Fig. 2I, J).”

Reviewer’s comment:

4) On page 9, lines 8-10, the authors claim that their number of cells was "sufficient" for single-cell analysis; the numbers are <500 for all samples. The authors need to justify this statement or articles that carefully analyze the number required for such a conclusion as references.

Response to Reviewer:

In the revised manuscript, we include the analysis of how many cells are needed to identify cluster of 6 cell types in this paradigm, based for example on the algorithms developed in our collaborative efforts (see Treppner et al. 2021). We determined statistical power for learning lower-dimensional representations of our scRNA-seq data using scVIDE (Treppner et al., 2022) as shown in the revised Appendix Fig. S5C, D. With both labeling approaches, the number and the quality of cells recovered were, as claimed, sufficient for subsequent scRNA-seq analysis. We refer to this figure/analysis in the main text on page 10:

“With both labelling approaches, the number and the quality of cells recovered were sufficient for subsequent scRNA-seq analysis (Appendix Fig. S5A-D).”

Further information on the analysis using scVIDE is found together with figures and the respective reference in the Appendix.

Reviewer’s comment:

5) The authors use Seurat and RaceID without their appropriate citations in the first mention during the results. The authors also stop immediately after DEG analysis along with clustering. The authors could analyze their RNA-seq data with a trajectory; to say the least, the identification/characterization of TTS and neurons as Neurons I, II, and III are insufficient. There could be multiple ways to show the "fate" of cells in the isolated FACS, which the authors have missed.

Response to Reviewer:

Based on the reviewer’s comment, the missing citations have been added in the results section. In the manuscript we refer to them on page 10:

“We used two different algorithms for clustering microinjected cells according to their transcriptomes. In Seurat-based clustering [38], progenitors appeared as one cluster in the microinjection data set (Fig. 5A), which could be separated in APs and BPs using RaceID clustering [39] in this data set (Fig. 5B).”

In regard to our extended characterisation of the TTS, please refer to the comment of reviewer 1 above; briefly: we now provide in the revised manuscript GO terms analysis for TTS vs BP and TTS vs Neurons (Fig. EV3A-C; see also Data Set EV1), predicted lineage trajectory using scvelo (Fig. EV4B), using Slingshot (Fig. EV4C), and using Monocle (Fig. EV4D).
We also analysed the co-expression of Eomes and Tubb3 in the TTS (Fig. EV4E).

Reviewer’s comment:
6) The authors detected candidates like Fgfr3, Nr2f1, Ofd1, and Mme as part of their treated (different approaches) datasets (from their DEG analysis). They correctly cite Huang et al., 2020 but fail to give us a sense of the consequences of these gene dysregulations. The authors can also validate if these proteins are expressed in their treated cells.

Response to Reviewer:
In the revised manuscript we refer to selected literature that report on phenotypes after misexpression of Ofd1, Mme, Fgfr3 and Nr2f1.
On page 11 we write:

“These genes can affect the balance between proliferation and differentiation [40-45].”

In the revised manuscript we also provide single molecule FISH staining to show the expression of Ofd1 and Nr2f1 upon EPZ treatment. We are providing this information in the revised Fig. EV2E.
We are referring to this figure on page 11:

“We exemplarily conducted single molecule FISH experiments using probes against Ofd1 and Nr2f1, which showed an increased expression upon DOT1L inhibition compared to controls (Fig. EV2E).”

Reviewer’s comment:

7) The authors list a few GO terms (page 10, lines 1-10) and associate them with reduced proliferation; they must cite relevant studies. The authors can also add supplementary data showing which genes in their data correspond to these GO terms.

Response to Reviewer:

We thank the reviewer for pointing out the missing citations. We now provide the missing citation and included an excel file as Data Set EV1 with the GO term enrichment analysis including the genes that are classified in the respective categories.
On page 11/12 of the revised manuscript we state:

“The data so far suggested that TTS’s transcriptional makeup described above had hallmarks of APs, but had at the same time a decreased proliferative capacity (for example reduced expression of Cdk-complex members [46] including Ccna2, Cdk1, Ccnb1, Cdk6, Cks2, Cks1b, Ccnb2, Ccnd2, Pcna, Ccnd1, Ccng2, Data Set EV1).”

Reviewer’s comment:

8) On Page 11, lines 3-7, the authors describe their method to arrive at the 17 targets with TF activity from the previous analysis. Can the authors describe the method used to correlate the two? The reviewer understands this could be MEME analysis or analysis of earlier datasets of Ferrari et al. 2020. But it must be explicitly stated, and a few examples in supplementary need to be exemplified as this analysis is key to discovering the three metabolic genes.

Response to Reviewer:

In the revised manuscript, we clarify the exact analysis that resulted in the identification of the 17 target genes, using the specific tool for gene network analysis. The analysis was based on a bioinformatics package called SCENIC.
We are describing its features in the material and methods on page 31:

“Single-cell regulatory network inference and clustering (SCENIC) analysis. Gene regulatory network activity (GRN) in each cell was computed with SCENIC as previously described [51]. SCENIC works in three steps: 1) prediction of transcription factors (TFs) and their target genes based on their co-expression in scRNA-seq data, 2) check for direct binding motifs of the TFs in the target genes and perform motif enrichment analysis using RcisTarget, and 3) activities of the resulting Gene Regulatory Networks (GRN) also called regulons are scored for each cell. This tool takes Seurat objects as input. Seurat objects were exported after clustering analysis of the electroporation dataset and divided into separate objects for control and EPZ cells, respectively. Default settings were used for building the co-expression network and creating regulons. The top 50% regulons were used in the GRN activity scoring. Heatmaps of the GRN activities per cell were generated using pheatmap (v1.0.12). Since each GRN is controlled by one TF, GRN activity and TF activity are used interchangeably here.

Selection of target genes. SCENIC outputs were explored further to narrow down on potential target genes of DOT1L activity in our data. The regulon analysis step outputs a table containing TFs, their target genes, predicted binding motifs, whether the TFs bind with high conformity, and a calculated Normalised Enrichment Score (NES). TFs with high conformity predicted binding motifs in their target genes was selected. Amongst the selected TFs, those showing changed patterns of activity upon DOT1L inhibition were extracted. The resulting list of genes were intersected with DEGs from each cluster and this list was kept as TF target genes with altered expression upon DOT1L inhibition.”

We modified the text according to the outline and provide now specific information on page 13:

“We performed unbiased analysis of (i) alterations of TF activities in gene regulatory networks, (ii) differentially expressed genes (DEGs) in APs, BPs and in the TTS, and (iii) GO terms enriched within DEGs, of electroporated cell scRNA-seq data. The analysis of changes in the TF network activity in response to DOT1L inhibition, in a cell-type resolved manner, was done using the SCENIC package [51], that revealed a set of key transcriptional regulators with treatment and cell-type specific activity changes (Fig. 6A, Fig. EV5A). Atf4, Cepbg, Ets1, 2, Ezh2, JunB, Pou3f1, Pou32f2, Rad21, Sox2, 4, 9, 11, 21, Tcf7l1 and Tcf4, which were present in both treatment conditions, changed activity upon DOT1L inhibition in APs, BPs or TTS. Intriguingly, analysis of GO terms that were enriched in APs and BPs revealed that the DEGs upon DOT1L inhibition included metabolism and oxidative phosphorylation (OxPhos), a hallmark of neuronal differentiation, suggesting thus a potential alternative mechanism compared to

spindle orientation (Fig. EV3D, E). The DEGs in the TTS also enriched for GO terms pointing towards metabolic changes, but also towards a more differentiative state upon DOT1L inhibition compared to control (Fig. EV3F, Appendix Fig. S6).

We next correlated TF with altered activity and their respective targets with altered expression levels upon DOT1L inhibition in our data set. We used four filtering steps that we applied to the SCENIC output: (i) identification of TF with opposite activities in APs compared to TTS, (ii) selection of predicted targets with a high motif conformation, (iii) intersection of putative targets from both control and EPZ condition that passed the first two filtering steps, and (iv) intersection of the TF targets with DEGs upon DOT1L inhibition in APs and TTS. This analysis retrieved 17 potential target genes, among which we found increased expression of three metabolic enzymes that are involved in producing intermediates that fuel, directly or indirectly, the TCA cycle, i.e. Asns, Gapdh, and Hk2 (Fig. 6B)."

3. Description of the revisions that have already been incorporated in the transferred manuscript

n/a

4. Description of analyses that authors prefer not to carry out

Reviewer's comment:

Tis21 data (1H), again of low quality, is only a single piece of evidence and the conclusion "suggesting that the acquisition of a basal fate was paralleled by a switch to neurogenesis" is premature. I think other cell cycle exit reporters, Fucci markers, pHis, BrdU, NeuroD, or Tbr2 reporters (Li et al., 2020, (Haydar and Sestan labs)) to name a few, are necessary to establish the conclusions. The authors should show other markers such as PAX6, EOMES, or other upper-layer markers upon cell cycle exit in the SVZ/CP. These additional experiments will assist in cell fate analysis.

Response to Reviewer:

We think establishing the Fucci or EOMES mice system is beyond the scope of the manuscript as it will not provide more information than the ones we obtained from systematic and extensive co-staining experiments.

In addition (and unfortunately) all labs involved are now facing a logistic issue (the animal house at HT is not ready yet and our license, in its present version, covers only the work with wt mice) that made importing and setting up of the colony unattainable for the next 6-10months.
If the reviewers and/or the editorial board think this is a major point compromising the entire revision, we kindly ask to contact us again so that we can discuss the issue and arrive to a shared conclusion.

Dear Elena,

Thank you for the submission of your revised manuscript. We have now received the enclosed reports from the referees. Referee 1 still has some more minor but important suggestions that I would like you to address and incorporate before we can proceed with the official acceptance of your manuscript. Please co-submit a point-by-point response with your final manuscript.

A few editorial requests will also need to be addressed:

- A callout to Appendix Table S1 is missing. This table might be better included in the main ms file as a Reagents and Tools table in the materials and methods.
In this case, it does not necessarily need to be cited in the ms text.

- The checklist for your SOURCE DATA is in, but the source data themselves are missing. Please upload the source data as 1 file per figure or per figure panel. The source data file can be zipped.

I would like to suggest some changes to the title and abstract that needs to be written in present tense. Please check the following text carefully and let me know whether you agree with the changes and whether all is correct:

DOT1L activity affects neural stem cell division mode and reduces differentiation and ASNS expression

Cortical neurogenesis depends on the balance between self-renewal and differentiation of apical progenitors (APs). Here, we study the epigenetic control of AP's division mode by focusing on the enzymatic activity of the histone methyltransferase DOT1L. Combining lineage tracing with single-cell RNA sequencing of clonally related cells, we show at the cellular level that DOT1L inhibition increases neurogenesis driven by a shift of APs from asymmetric self-renewing to symmetric, neurogenic consumptive divisions of APs. At the molecular level, DOT1L activity inhibits [OK?] AP differentiation by promoting transcription of metabolic genes. Mechanistically, DOT1L inhibition reduces the activity of an EZH2/PRC2 pathway, converging on increased expression of Asparagine synthetase (ASNS), a microcephaly-associated gene. Overexpression of ASNS in APs phenocopies DOT1L inhibition, and also increases neuronal differentiation of APs. Our data suggest that DOT1L activity/PRC2 crosstalk controls AP lineage progression by regulating Asparagine metabolism.

The synopsis image is OK, but we also need a short summary and bullet points for the EMBO reports website. Can you please send us A) a short (1-2 sentences) summary of the findings and their significance, and B) 2-3 bullet points highlighting key results. Thank you.

I look forward to seeing a final version of your manuscript as soon as possible. Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Best wishes,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

Most of the raised points were revised either by new experiments or additional explanations. Overall, the quality of the manuscripts is fairly improved. But there are still some unclear parts/discrepancies, and the authors should explain these parts. It can be only published after all these minor but important points are properly revised. Please see the following points

-Point1: New FigEV1
"To further investigate the effect of DOT1L inhibition on the proliferative or neurogenic potential of cortical progenitors, we first assessed the number and distribution of mitotic, pHH3 positive cells (Fig. EV1E). We observed no difference in the number of pHH3 positive cells, nor in the distribution of pHH3 positive cells between the VZ (apical localisation) and SVZ (basal localisation) (Fig. EV1F). These data suggest that the increased neurogenesis observed upon EPZ treatment was not due to a general anti-mitotic effect of the drug."

Logically, I do not understand this data. In the figure 1, EPZ-treatment induced delamination, and in Fig2B, total EdU+ cells (both Sox2+ and Eomes+ population) were decreased, indicating the reduction of proliferating cells. Now, with the new data with pHis-staining, the authors stated that EPZ-treatment has no effects on proliferation in general. How do they explain this

discrepancy? This is also inconsistent with their conclusion from scRNA-seq data.

-Point2: Fig2I/2J
Please indicate the degree of Dot1l-KD
Please show the high magnification images around SVZ. It is difficult to see whether KD of Dot1l induced Ctip2/TUj1 expression. It rather looks like that shDot1 induced the Tubb3 expression in non-GFP positive cells.
Some bar graphs are missing the error bars?

-Point3: Fig2C.
"In the revised manuscript, we provide a clarification on the criteria of Tis21-GFP+ signal thresholding. In the revised Fig. 1H (now Fig. 2C, right part) we now indicate with arrows pointing to the respective cells the signal thresholding for the Tis21-GFP signal.
We are referring to the classification as well in the respective figure legend on page 34/35:
"C) Immunofluorescence staining showing overview of TIS21-GFP-positive (GFP+) cells in VZ of control and EPZ treated sections. Scale bars: 10 μm. Magnifications on the right show exemplarily a TIS21-GFP positive cell (arrow), mild positive cell (arrow with dashed line), and a negative cell (arrowhead)."

Unfortunately, if I did not mistake, I could not find a proper explanation regarding the definition of GFP+, mild-GFP+, and negative cells. What the authors indicates is only subjective definition with arrows/arrowheads, but it is totally unclear how the levels of GFP levels are quantitively defined. On the page 34/35 in the combined manuscript, I did not find any methodological explanation. I also checked the entire MM section, but I did not find it. Please explain how the intensity of GFP was measured and how each class of cells were quantitatively defined based on the GFP intensity. I would suggest the authors to take this seriously. Without clear definition, Fig2D does not support their statements, as all data seems to be generated based on their subjective definition, and it would not be possible to recapitulate.
If the authors have been using only subjective quantification methods for defining "positive" cells for markers, this should be improved in this study and future. Especially, the study is trying to define a new transient cellular state. Therefore, it is quite important to quantitatively compare how this cellular state is different from other state (Aps, BPs or neurons) using quantitative measurements.

-Point4: Fig3
"Having hence successfully implemented AP microinjection, we applied it to study at the singlecell level the effect of DOT1L inhibition on cell morphology, cell identity and lineage progression (Fig. 3A-F). We first analyzed the general composition of the progeny of microinjected cells and found that EPZ treatment did not change the proportion of cells found in two-cell clusters as opposed to single-cells (Fig. 3B, C). These data suggest that EPZ does not have nonspecific effects on cell cycle progression per se, nor that it causes mitotic arrest (see also data on pHH3 distribution). The morphological analysis showed that the inhibition of DOT1L resulted in an increase in the number of cells without apical contact, lacking the typical bipolar morphology of APs (Fig. 3B, D) suggesting the generation of a delaminated and more differentiated cell progeny (either BPs or neurons) compared to APs."

Again, please explain the discrepancy between EdU experiment and this statement.

-Point5, FigEV5
ChIP-qPCR for Dot1 at the ASNS promoter. Is it significant compared with IgG? If not, the statement is wrong.

Referee #2:

We have studied the revised manuscript by Appiah et al. We originally commented that the conclusions regarding increased neurogenesis following DOT1L inhibition were premature and had specific reservations regarding the data presented and the quality of the image. We feel that the authors added significant new data to support their observation, and we are satisfied with their response. Our other concern was the size of the single-cell analysis and validation of the data. The authors claim the quality of the recovered cell was sufficient, provided more details on the analysis that was presented, and supported their data with FISH experiments. Based on these, we recommend accepting the manuscript for publication.

Point by point response to Reviewers

We thank the reviewers for their valuable comments regarding our manuscript. We took all comments into account, and we are now presenting the revised version of our manuscript, including new data corresponding to all the experiments and analysis requested by the reviewers.

Manuscript number: RC-2022-01528 / EMBOR-2022-56233V2
Corresponding author(s): Elena Taverna and Tanja Vogel

Editorial requests:

Editorial comment:
A callout to Appendix Table S1 is missing. This table might be better included in the main ms file as a Reagents and Tools table in the materials and methods.
In this case, it does not necessarily need to be cited in the ms text.

Response to editorial comment:
We have now included the Appendix Table S1 as "Reagents and Tools" in the material and methods.

Editorial comment:
The checklist for your SOURCE DATA is in, but the source data themselves are missing. Please upload the source data as 1 file per figure or per figure panel. The source data file can be zipped.

Response to editorial comment:
The Source Data Files seem to have been lost in the submission process. They were added to the platform and the correct upload was confirmed before resubmission.

Editorial comment:
I would like to suggest some changes to the title and abstract that needs to be written in present tense. Please check the following text carefully and let me know whether you agree with the changes and whether all is correct:

DOT1L activity affects neural stem cell division mode and reduces differentiation and ASNS expression

Cortical neurogenesis depends on the balance between self-renewal and differentiation

of apical progenitors (APs). Here, we study the epigenetic control of AP's division mode by focusing on the enzymatic activity of the histone methyltransferase DOT1L. Combining lineage tracing with single-cell RNA sequencing of clonally related cells, we show at the cellular level that DOT1L inhibition increases neurogenesis driven by a shift of APs from asymmetric self-renewing to symmetric, neurogenic consumptive divisions of APs. At the molecular level, DOT1L activity inhibits [OK?] AP differentiation by promoting transcription of metabolic genes. Mechanistically, DOT1L inhibition reduces the activity of an EZH2/PRC2 pathway, converging on increased expression of Asparagine synthetase (ASNS), a microcephaly-associated gene. Overexpression of ASNS in APs phenocopies DOT1L inhibition, and also increases neuronal differentiation of APs. Our data suggest that DOT1L activity/PRC2 crosstalk controls AP lineage progression by regulating Asparagine metabolism.

Response to editorial comment:
We took up most of the proposed changes and now provide with the new manuscript a revised title and abstract.

Editorial comment:
The synopsis image is OK, but we also need a short summary and bullet points for the EMBO reports website. Can you please send us A) a short (1-2 sentences) summary of the findings and their significance, and B) 2-3 bullet points highlighting key results. Thank you.

Response to editorial comment:
We provide a new graphical abstract. The requested text additions are the following:

A) The epigenetic modifier DOT1L represses expression of Asparagine synthase to preserve the apical progenitor state and prevents microcephaly by premature neuronal differentiation.
B)

- DOT1L influences the balance between symmetric and asymmetric cell division
- DOT1L prevents expression of ASNS in apical progenitors in concert with PRC2
- DOT1L regulates apical progenitor metabolism

Reviewer #1
Reviewer's comment:
Most of the raised points were revised either by new experiments or additional explanations. Overall, the quality of the manuscripts is fairly improved. But there are still some unclear parts/discrepancies, and the authors should explain these parts. It can be

only published after all these minor but important points are properly revised. Please see the following points

Response to Reviewer:
We hope we have addressed all points raised by the reviewer in a satisfactory manner.

Reviewer's comment:
-Point1: New FigEV1
"To further investigate the effect of DOT1L inhibition on the proliferative or neurogenic potential of cortical progenitors, we first assessed the number and distribution of mitotic, pHH3 positive cells (Fig. EV1E). We observed no difference in the number of pHH3 positive cells, nor in the distribution of pHH3 positive cells between the VZ (apical localisation) and SVZ (basal localisation) (Fig. EV1F). These data suggest that the increased neurogenesis observed upon EPZ treatment was not due to a general anti-mitotic effect of the drug."
Logically, I do not understand this data. In figure 1, EPZ-treatment induced delamination, and in Fig2B, total EdU+ cells (both Sox2+ and Eomes+ population) were decreased, indicating the reduction of proliferating cells. Now, with the new data with pHis-staining, the authors stated that EPZ-treatment has no effects on proliferation in general. How do they explain this discrepancy? This is also inconsistent with their conclusion from scRNA-seq data.

Response to Reviewer:
We thank the reviewer for pointing out this flaw in our manuscript text.
We have accordingly revised our statements and also include that this observation (fewer cells in S-phase but similar numbers of M-phase cells) was also made upon the genetic deletion of Dot1l (Franz et al.). We explain in more detail on page 7/8:

"We studied the effect of DOT1L inhibition by labelling cycling cells at the end of 24 h HeRo culture with a 1 h EdU pulse, and analysed the number of proliferating cells as well as the fate of EdU-labelled cells by assessing SOX2 and EOMES expression. We observed an overall reduced number of cycling, EdU-positive cells and equal numbers of single SOX2+ or EOMES+ cells. This finding suggests that the DOT1L inhibition for 24 h impaired acutely S-phase entry or progression, but that the time frame was too short to impact overall numbers of APs or BPs. SOX2+/EOMES+ double positive cells increased upon DOT1L inhibition compared to controls indicating an increased transition of APs towards a differentiative fate (**Fig. 2A, B**). According to the reduced number of actively cycling EdU+ cells, we observed that b oth SOX2+/EdU+ and EOMES+/EdU+ double positive cells decreased upon DOT1L inhibition compared to controls (**Fig. 2A, B**), suggesting AP cell cycle exit and

acquisition of a differentiative fate, possibly a shorter presence of BPs, or impaired generation of BPs. As SOX2+/EOMES+ cells increased, the latter scenario seemed less likely compared to BPs progressing with differentiation upon DOT1L inhibition. We further investigated the effect of DOT1L inhibition on cell cycle progression of cortical progenitors by assessing the number and distribution of pHH3 positive mitotic cells (M-phase) (**Fig. EV1E**). We observed neither a difference in the number of pHH3 positive cells, nor in the distribution of pHH3 positive cells between the VZ (apical localisation) and SVZ (basal localisation) (**Fig. EV1F**). In summary, we observed that upon DOT1L inhibition fewer APs entered S-phase, and that after cell cycle progression they adopted a more differentiating fate. Since we observed APs in S- and M-phase as well as in a subsequent differentiating BP state, we excluded a general anti-mitotic but assumed a differentiative effect of the drug. Further, the observation of similar numbers of APs in M-phase but reduced numbers in S-phase suggested a possible alteration in the mode of mitosis, similar to our observations upon genetic loss-of-function of DOT1L (Franz *et al*, 2019)."

Reviewer's comment:
Point2: Fig2I/2J
Please indicate the degree of Dot1l-KD
Please show the high magnification images around SVZ. It is difficult to see whether KD of Dot1l induced Ctip2/TUj1 expression. It rather looks like that shDot1 induced the Tubb3 expression in non-GFP positive cells.
Some bar graphs are missing the error bars?

Response to Reviewer:
To indicate the degree of Dot1l-KD we now provide two quantitative and qualitative traits

- RT-qPRC: In the newly added Fig. EV1G we show RT-qPCR of N2a cells transfected with the shRNA compared to the shCtrl.
- Immunoflourescence. As described in our previous response to reviewers, the DOT1L antibody from Cell Signalling has never worked in our hands for immunofluorescence. We took care of testing and optimizing other Abs, and we found an anti-DOT1L Ab from Cell Atlas that works for immunofluorescence in tissue.
  We therefore used it to assess the degree of KD on electroporated cells.
  Fig. EV1H depicts DOT1L levels in control-electroporated and shDot1l-electroporated tissue. Both tissues contain both GFP-positive and GFP-negative cells. We then quantify the signal intensity using a line plot tool from FiJi, and drew a line crossing multiple cells, GFP-positive, as well as GFP-negative. The

line plot quantifications show that in Con-electroporation, the DOT1L signal is the same in all cells. In contrast, in shDTO1L-electroporated cells, the DOT1L signal is lower in GFP-positive cells compared to GFP-negative cells.

We write on page 8 of the revised manuscript:
"The efficiency of the DOT1L KD was shown in N2a cells and in *in utero* electroporated tissue (**Fig. EV1G, H**)."

We now show high magnification images around SVZ in Fig. 2I to illustrate that KD of Dot1l induced Ctip2/TUj1 expression as quantified in Fig. 2J.

The error bars are missing as in these cases all replicates have the same value, which can be corroborated looking at the Source Data Files.

Reviewer's comment:
Point3: Fig2C.
"In the revised manuscript, we provide a clarification on the criteria of Tis21-GFP+ signal thresholding. In the revised Fig. 1H (now Fig. 2C, right part) we now indicate with arrows pointing to the respective cells the signal thresholding for the Tis21-GFP signal. We are referring to the classification as well in the respective figure legend on page 34/35: "C) Immunofluorescence staining showing overview of TIS21-GFP-positive (GFP+) cells in VZ of control and EPZ treated sections. Scale bars: 10 µm. Magnifications on the right show exemplarily a TIS21-GFP positive cell (arrow), mild positive cell (arrow with dashed line), and a negative cell (arrowhead)."

Unfortunately, if I did not mistake, I could not find a proper explanation regarding the definition of GFP+, mild-GFP+, and negative cells. What the authors indicates is only subjective definition with arrows/arrowheads, but it is totally unclear how the levels of GFP levels are quantitively defined. On the page 34/35 in the combined manuscript, I did not find any methodological explanation. I also checked the entire MM section, but I did not find it. Please explain how the intensity of GFP was measured and how each class of cells were quantitatively defined based on the GFP intensity. I would suggest the authors to take this seriously.
Without clear definition, Fig2D does not support their statements, as all data seems to be generated based on their subjective definition, and it would not be possible to recapitulate.
If the authors have been using only subjective quantification methods for defining "positive" cells for markers, this should be improved in this study and future. Especially, the study is trying to define a new transient cellular state. Therefore, it is quite important

to quantitatively compare how this cellular state is different from other state (Aps, BPs or neurons) using quantitative measurements.

Response to Reviewer:
The GFP signal was measured following previously established protocols (Haubensak et al., Wong et a.l, Arai et al.), that are now described in material and methods and that we now cite in the relevant section for clarity.

We added the following explanation to material and methods on page 27:
"The cell's neurogenic potential was assessed by scoring cell's positivity to GFP in the *Tis21-Gfp* mouse neocortices (Haubensak *et al*, 2004; Wong *et al*, 2014). For the quantification of immunofluorescence intensity levels, the area of the nucleus of interphase cells was selected using the DAPI staining as a guide (Wong *et al.*, 2014). Cells positive for GFP were marked as TIS21-GFP+ and those negative for it marked as TIS21-GFP-. For the TIS21-GFP+ positive cells, the GFP positivity was classified as faint if the intensity was in the lower 30% portion of the GFP positivity range. Proportions of TIs21-GFP+ strong, TIS21-GFP+ mild and TIS21-GFP- were calculated as percentage of total cells per condition. ."

We acknowledge the points raised by the reviewer regarding signal intensity quantification, and we think that a fully automated, possibly AI-based image analysis tool would be the only way to obtain a truly unbiased and objective measure of the GFP intensity and might therefore be a useful suggestion to improve these quantifications (and quantifications of others in the field, that are equally scoring based on positivity) in future studies. However, we think developing such a tool is beyond the scope of this work. We therefore employed the quantification protocol (including thresholding) that was previously developed and extensively used for scoring Tis-21-GFP positivity (see Haubensak et al.; Kosodo et al.; Fei et al.; Arai et al.; Attardo et al.; Wong et al.; Taverna et al; Mora-Bermudez et al.; Calegari et al. and other publications from Huttner lab) .

Regarding in particular the work we present here, we do not agree that the protocol we employed to discriminate between strong and mild GFP-positive signals would hamper recapitulation of the data presented in our study; neither that it would result in a different observation other than DOT1L inhibition activating a neurogenic fate of progenitor cells. The fraction of cells in question is the mild positive cells - that could either be false positive or false negative. In either case, doing a statistical test on {negative + mild positive} vs. {posititve}, or on {negative} vs {mild positive + positive} returned statistical significant differences between the two conditions, EPZ vs control.

This shows that our interpretation of the effect of DOT1L inhibition on initiating neuronal differentiation is independent of the (possible) subjective steps in our quantifications.

Of note, we do not claim in our manuscript that the transient cell state corresponds to Tis21-expression levels. We are aware that this would be an overinterpretation of our data, which is why we refrained from it.
However, we provide solid quantitative assessment of this cell fraction through our single-cell RNA sequencing data (that are totally independent from the Tis-GFP experiment). Until now, we do not have other tools at hand which we could use to unequivocally quantify the transient cells *in vivo*, because all markers we tested so far pick up also other cell populations. We think this might be in line with the idea that we are referring to a cell state, rather than to a separated cell population, expected to have its own specific transcriptional signature.

Reviewer's comment:
Point4: Fig3
"Having hence successfully implemented AP microinjection, we applied it to study at the single cell Dot1L inhibitors level the effect of DOT1L inhibition on cell morphology, cell identity and lineage progression (Fig. 3A-F). We first analyzed the general composition of the progeny of microinjected cells and found that EPZ treatment did not change the proportion of cells found in two-cell clusters as opposed to single-cells (Fig. 3B, C). These data suggest that EPZ does not have nonspecific
effects on cell cycle progression per se, nor that it causes mitotic arrest (see also data on pHH3 distribution). The morphological analysis showed that the inhibition of DOT1L resulted in an increase in the number of cells without apical contact, lacking the typical bipolar morphology of APs (Fig. 3B, D) suggesting the generation of a delaminated and more differentiated cell progeny (either BPs or neurons) compared to APs."
Again, please explain the discrepancy between EdU experiment and this statement.

Response to Reviewer:
We have rephrased this finding in the revised manuscript to make clear the difference between overall capacity of cell to conduct cell division upon DOT1L inhibition alongside potential effects of the drugs on specific phases of the cell cycle, i.e. M-phase.
The similar numbers of Eomes upon acute EPZ treatment are in accordance with the EdU experiment. The reason for seeing unchanged Eomes expressing cells might be the duration of the inhibition or a specific time dynamics of generation/differentiation of the BPs.
We now write on page 9 of the revised manuscript:

" Having hence successfully implemented AP microinjection, we applied it to study at the single-cell level the effect of DOT1L inhibition on cell morphology, cell identity and lineage progression (**Fig. 3A-F**). We first quantified the progeny of microinjected cells and found that EPZ treatment did not change the proportion of cells found in two-cell clusters as opposed to single-cells (**Fig. 3B, C**). These data suggest again that EPZ did not arrest cells in the cell cycle but principally allowed cell division (**Fig. EV1F**) . The morphological analysis showed that the inhibition of DOT1L resulted in an increase in the number of cells without apical contact, lacking the typical bipolar morphology of APs (**Fig. 3B, D**) suggesting the generation of a delaminated and more differentiated cell progeny (either BPs or neurons) compared to APs. Staining with EOMES and TUBB3 revealed no change in the fraction of EOMES-expressing cells (BPs; **Fig. 3B, E**), compared to a strong increase in the proportion of TUBB3-expressing cells (neurons; **Fig. 3B, F**). Taken together, these data suggest that the inhibition of DOT1L might favour APs to acquire a differentiative fate that results in the generation of increased numbers of neurons."

Reviewer's comment:
Point5, FigEV5
ChIP-qPCR for Dot1 at the ASNS promoter. Is it significant compared with IgG? If not, the statement is wrong.

Response to Reviewer:
We increased the n number for this ChIP-qPCR experiment and switched to neural progenitor cells instead of cortical tissue, to show that the DOT1L protein enriches significantly at the ASNS promoter region. The new figure has been shifted from the EV5D figure into the main figures as Fig. 6G.

Dr. Elena Taverna
Human Technopole
Viale Rita Levi-Montalcini, 1
Milan 20157
Italy

Dear Elena,

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Senior Editor
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Corresponding Author Name: Prof. Dr. Tanja Vogel, Dr. Elena Taverna
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Abridged guidelines for 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.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots 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. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship 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.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Not Applicable	

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	See Appendix

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Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Material and Methods, Appendix

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Yes	Material and Methods
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Not Applicable	
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

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Animal observed in or captured from the field: Provide species, sex, and age where possible.	Not Applicable	
Please detail **housing and husbandry conditions**.	Not Applicable	

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Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
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If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

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If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	See Acknowledgements section

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Yes	We refer to the relevant litarature in the main text

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	See relevant section in the main text
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Yes	See material and methods section
Include a statement about **blinding** even if no blinding was done.	Yes	See material and methods section
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	See material and methods section

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Legends
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Legends

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: 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	
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Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	See material and methods section
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
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Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

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State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
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	
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Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	material and methods
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Yes	material and methods
If publicly available data were reused, provide the respective **data citations in the reference list**.	Yes	material and methods