PAR3 restricts the expansion of neural precursor cells by regulating hedgehog signaling

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Original submission

First decision letter

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MS TITLE: PAR3 Restricts the Expansion of Neural Precursor Cells by Regulating Hedgehog Signaling

AUTHORS: Tomonori Hirose, Yoshinobu Sugitani, Hidetake Kurihara, Hiromi Kazama, Chiho Kusaka, Tetsuo Noda, Hidehisa Takahashi, and Shigeo Ohno

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. The referees raise two major issues that they would like addressed. First, improved characterisation of apicobasal polarity and adhesion to better define the basis for ectopic mitoses in the mutant. Second, strengthening the data related to Shh signaling in vivo. Assaying Smo cilia localisation in vivo would help and carefully documenting dorsal ventral locations of the analyses and any effect on patterning (as indicated by Referee 2).

If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy to receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and

where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary. Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The manuscript by Hirose et al explores the role of the apico-basal polarity regulator Par3 in early neural development in the mouse cortex.

The authors generated a conditional null allele to circumvent the midgestational death of homozygous mutants observed in the constitutive KO strain, and characterize the phenotype of a forebrain specific deletion. They use an early driver (FoxGI-Cre), and claim that the defects they describe in the present study are specific to early stages, as they are not reproduced using a later driver (Nestin-Cre)

The focus of the study is therefore on early stages of cortical development, centered on the transition from pure amplification of the pool of progenitors to neurogenic divisions.

The paper starts by describing a disorganized cortex, showing ectopic mitoses, rosettes and misplaced neurons, and a slightly enlarged cortex. Ectopic progenitors may result from a loss of planar spindle orientation (however the defects are modest and I find it unlikely that they would by themselves result in such a disorganization). The enlargement is then attributed to an increase of proliferation at the expense of differentiation.

The authors then use candidate screening by Q-RT PCR to identify signaling pathways that may be dysregulated in the early cortex. The Notch pathway target Hes5 is enriched, but further biochemical exploration of Notch pathway players fails to confirm a strong dysregulation of the pathway (and indeed, the enrichment in progenitors over neurons described earlier may suffice to explain the increase in Hes5 transcripts, although the authors do not propose this interpretation).

They also identify an increase in activation of the Shh pathway, an confirm in a neurosphere culture pathway that Par3 inactivation leads to an increase in the expression of downstream Shh pathway components, which can be counteracted by treatment of the culture by a Smo antagonist, leading to the proposal that Par3 inhibits Shh pathway activation and self-sustained activity to restrict proliferation in progenitors.

Exploring this hypothesis, they show defects in primary cilium morphology in the mutants as early as E10.5, suggesting that Shh signal may not be properly received and processed in the mutants. Switching to in vitro reporters of the shh pathway in 3T3 cells, they find that while the localization of Ptch-YFP to the cilium of serum-starved 3T3 cells is not modified by down-regulation of Par3, the localization of YFP-Smo increases upon Par3 knock-down. This leads to a model in which Par3 restricts Smo localization in the cilium by regulating ciliary integrity, therefore limiting Shh signaling.

Overall, the message of the paper is clear and novel, and offers a fresh view on the role of Par3 in neurodevelopment.

Comments for the author

While the overall interpretation fits with the data, there are several weaknesses in this paper, that should be addressed in order to make it fit for publication.

1) The effects on apico-basal polarity and adhesion is insufficiently characterized: Although the authors do not detect major defects in polarity markers at E10.5, the overall defects in organization is striking. It is unclear whether ectopic mitoses are due to loss of adhesion (a very common source of ectopic mitoses in the literature), to misoriented divisions or to failure to complete proper INM (which seems to be the authors preferred hypothesis)? In any case, it seems unlikely (but not impossible) that it can be explained solely by defective spindle orientation, as the defects are relatively modest. The nestin and vimentin stainings (Fig3) suggest stronger disorganization. This should be further characterized. A membrane labeling (eg through in utero electroporation of a mosaic mb-GFP) to characterize cell shape, and in particular the shape and adhesions of ectopically dividing cells, would help resolve the issue. Live imaging of dividing cells (either in sections or in en-face views) would also show whether spindle orientation defects are indeed causing delamination.

2) The characterization of Smo and Ptch localization is performed in vitro in 3T3 cells. Cilium formation in these cells is triggered by serum starvation, independent from cell junctions and apico-basal polarity. It is not clear whether this represents a good model for the very polarized and organized neuroepithelium; even if the data in this model nicely fit with the rest of the observations in the mutants, this results in an overall interpretation that is constructed from completely independent systems. As there are some antibodies to label Smo, a characterization of its localization in the Par3 mutant cortex appears feasible and would provide a much more convincing result to support the authors' model.

Minor remarks:

Figure 6 G-I: higher magnification images and separate color channels would be preferable, as it is nearly impossible to see the relevant signals on these images.

A number of data throughout the manuscript are indicated as "not shown", it would be good to show the data (eg: "stimulation with 1 or 10 nM SAG1.3, a direct agonist 9 of Smo (Chen et al., 2002), induced greater accumulation of YFP-Smo … (not shown)")

In the discussion, the authors state that "Here, we demonstrate that PAR3 is required for this appropriate transition by restricting proliferative NPC divisions in early telencephalic development". Not exactly: actually, what we are seeing is both increased and ectopic proliferation and we cannot judge whether or not this increase is a consequence of a possible apical detachment…

Discussion lines14-16
"Although we also examined the telencephalon of mice with conditional deletion of PAR3 with the Nestin-Cre transgene (NesCreTg+; Par3ΔE3/floxE3) in the early neurogenic phase around E11.5 (Fig. S2C,D) (Imai et 15 al., 2006), it exhibited much milder phenotypes than the Par3 cKO (Foxg1+/Cre; Par3ΔE3/floxE3) telencephalon (Fig. S2E–S2J)."

This sentence is very confusing: S2CD is first mentioned and does correspond to an early stage (E11.5) but S2E-J are E12.5-E14.5, and do not show an obvious phenotype while FoxG1Cre-Par3cKO show a strong phenotype from 11.5. The obvious interpretation is that Par3 KO is deleterious only when it takes place at early stage. This could could be stated more explicitly, and the difference in expression onset between FoxG1 and Nestin could also be explained more explicitly.

Reviewer 2

Advance summary and potential significance to field

In their manuscript, Hirose et al., investigate the molecular mechanisms that underlie the switch from symmetric proliferative to asymmetric neurogenic divisions in neural stem cells. To this end, they generated and characterized Par3 conditional mouse mutants in which Par3 is specifically inactivated in the telencephalon at the time this switch occurs. They found that the Par3 mutation leads to increased neural progenitor proliferation and the formation of an expanded subventricular zone. On a cellular level, these changes coincided with alterations in interkinetic nuclear

migration, in the orientation of the mitotic spindle and most notably in the morphology of the primary cilium. Consistent with ciliary defects, they also detected increased Shh signalling and showed increased Smo ciliary localisation in 3T3 Par3 knock-down cells.
Investigating the mechanisms they lie at the basis of neural stem cells to switch to neurogenic cell divisions is of great importance as it determines the size of the neural stem cell pool and ultimately the size of the developing brain. Alterations in this switch are at the centre of the evolutionary expansion of the human brain and of several neurodevelopmental disorders such as microcephaly and macrocephaly. On the other hand, very little is known how this crucial developmental step is controlled. Hence, this manuscript addresses an important, open question in Neurodevelopmental Biology and I recommend the publication of this manuscript, however, there are a number of major points which need to be addressed first.

Comments for the author

1) Par3 is inactivated in the dorsal as well as the ventral telencephalon. Neural stem cells in these two structures differ very much in their proliferation and differentiation characteristics but the authors do not seem to distinguish between these two regions. It rather appears that some analysis were performed in the ventral telencephalon, others with the dorsal telencephalon or even with whole telencephalic extracts. The manuscript would greatly benefit from a separate analysis of dorsal and ventral telencephalon. This is particularly important given the proposed role of increased Shh signalling. Whereas this pathway is active in the ventral telencephalon under physiological conditions, it is inactive in the dorsal telencephalon. Therefore, the model the authors propose that the expansion phase is characterized by high levels of Shh signalling does not apply to dorsal telencephalic progenitors.

2) An important caveat the authors need to address refers to a potential patterning defect caused by the enhanced Shh signalling. A ventralisation of dorsal progenitors could cause dramatic changes in their proliferation/differentiation characteristics. To this end, they need to carefully investigate the expression of ventral and dorsal progenitor and neuron markers in the Par3 mutant.

3) The authors investigate the state of Shh signaling but their data is not very convincing on this point. While they note increased Gli1 expression, the expression of Ptch1, a commonly used marker of Shh signalling, is not altered. The authors do not comment on this difference. Moreover, primary cilia are not only involved in mediating the activation of Shh signalling but also in the formation of the Gli3 repressor. The authors need to analyse the levels of Gli3 activator and repressor forms and the ratio between the two forms in the dorsal and ventral telencephalon separately. They should also compare the Par3 phenotype with that of Gli3 conditional mutants, in which the switch to neurogenic divisions is delayed in the dorsal telencephalon. They should also discuss the role of primary cilia in telencephalic progenitors as revealed by several mouse mutants.

4) The two main findings of the manuscript, increased neural stem cell proliferation and increased Shh expression, are not linked by experiments. The authors need to perform either genetic or pharmacological rescue experiments. For example, they could cross Par3 mutants with Smo mutants or treat Par3 mutants with cyclopamine to inhibit Shh signalling.

5) The identity of the progenitors in the ectopic SVZ needs to be clarified by staining for Tbr2 (intermediate progenitors) or for Hopx (outer radial glial cells). Alternatively, these cells could have required a ventral fate (see point 2). This could be investigated with Gsx2, Dlx2 or Ascl1 stainings.

6) Many experiments are not or only inappropriately quantified. Experiments with lack of quantification: Fig. 1D; Fig. 2; Fig. 4B, D; Fig. 5B. Figure 3D and F only use one embryo and two embryos, respectively. Therefore, n should be 1 and 2, respectively. The authors need to increase the number of analysed embryos to at least three and compare the averages from each embryo to avoid pseudo-replication. Statistical significance needs to be indicated for all experiments which, for example, is missing in Fig. 6C, H and J. Without this information, it is impossible to judge the outcome of these experiments.

First revision

Author response to reviewers' comments

First, we mainly agree with the comments on the following three major issues to be addressed as suggested by you and the reviewers.

1. Improved characterization of apicobasal polarity and adhesion to better define the basis for ectopic mitoses in the mutant.
2. Strengthening the data related to Shh signaling *in vivo*.
3. Carefully documenting dorsal ventral locations of the analyses and any effect on patterning.

For the first issue, in addition to the careful characterization of cell shape by staining for nestin and phospho-vimentin (pSer55), we stained for pericentrin and beta-catenin as markers of apicobasal polarity and adhesion. However, it was not feasible for us to observe dividing NPCs under live imaging conditions as suggested by Reviewer 1. To observe the shape of *Par3* cKO NPCs, it would be necessary to electroporate an mb-GFP vector *in utero* at approximately E9.0, before or just after the onset of Cre expression from the *Foxg1Cre* allele. This is obviously technically impossible for us. Although live imaging using a suitable transgene might be another choice, it is very time consuming because it is necessary to prepare mice on a 129SvJ background to ensure the tissue specific Cre expression from the *Foxg1Cre* allele (Hébert et al. Dev Biol. 2000). These major difficulties prevented us from trying live imaging.

For the second issue, we optimized the fixative and staining conditions for the detection of ciliary Smo by whole-mount double immunofluorescence using anti-Smo mAb and anti-IFT88 pAbs as a ciliary marker (from page 12, line 372). To assess whether the hyperactivation of hedgehog signaling was responsible for the phenotypes observed in *Par3* cKO telencephalons, we injected cyclopamine, as suggested by Reviewer 2, into pregnant dams to inhibit Smo activity *in vivo* and analyzed the dorsal telencephalons of embryos (page 12, lines 377–379). We quantified the numbers and distribution of dividing NPCs in three pairs of *Par3* cKO telencephalons obtained from pregnant dams treated for 24 hours with 10 mgl/kg cyclopamine or vehicle, 45% 2-hydroxypropyl-beta-cyclodextrin in PBS(–) (Fig. 7D–F).

For the third issue, we carefully analyzed the dorsal and ventral parts of telencephalons separately to extend the initial data: e.g. the shape of NPCs, quantification of the distribution of dividing NPCs, and the fate of divided NPCs (Figs. 3A–J, 4A–C, 4H–J). Dorsoventral patterning of the telencephalon was evaluated in E10.5 or E11.5 embryos by immunofluorescence of Shh and Nkx2.1, Pax6 and Gsh2, and immunohistochemistry for Ascl1 and Tbr2 (Fig. S4). For western blotting using anti-Gli3 pAb, we prepared telencephalons at E11.5 by separately dissecting them into dorsal and ventral parts (Fig. 5E). The successful separation of these parts was further confirmed by western blotting using anti-Nkx2.1 pAb as a ventral marker protein (Fig. S3).

4. **Other improvements.**

For the analysis of interkinetic nuclear migration, we included one more pair of *Par3* cKO and control embryos at E10.5 and E11.5 each (Fig. 3K–N). To explicitly indicate the embryos analyzed for ectopic divisions and cleavage orientation of NPCs, data from different embryos are shown in different colors (Fig. 4B, C, E, F). The results of western blotting were analyzed quantitatively and shown as bar graphs (Fig. 5B, D, F; Fig. S3B).

Responses to Reviewer 1:

1) The effects on apicobasal polarity and adhesion are insufficiently characterized:

REPLY: To assess these characteristics, we performed immunofluorescence staining of pericentrin and beta-catenin in Fig. 3A-H (from line 190 on page 7). These data revealed that many ectopically dividing *Par3* cKO NPCs had rearranged cell-cell junctions away from the ventricular surface. Immunofluorescence of p-vimentin also demonstrated some *Par3* cKO NPCs in the outer region had lost apical domains but had extended basal processes to the pial surface at E11.5 (Fig. 3H). Together with the defective interkinetic nuclear migration and misoriented divisions, we consider that the perturbed apicobasal polarity and cell-cell junctions might cause the histological disorganization of the *Par3* cKO

telencephalon (page 8, lines 224–226).

2) *The characterization of Smo and Ptch localization is performed in vitro in 3T3 cells. Even if the data in this model nicely fit with the rest of the observations in the mutants, this results in an overall interpretation that is constructed from completely independent systems. As there are some antibodies to label Smo, a characterization of its localization in the Par3 mutant cortex appears feasible and would provide a much more convincing result to support the authors' model.*

REPLY: In accordance with this suggestion, we analyzed ciliary Smo in the *Par3* cKO telencephalon by whole-mount immunofluorescence for Smo and IFT88 at E11.5 (Fig. 7A, B) (from page 12, line 372). We detected significantly higher ciliary Smo accumulation in the dorsal and ventral *Par3* cKO telencephalons compared with controls. These data further support our model that PAR3 restricts the ciliary accumulation of Smo in NPCs (page 12, lines 375–376).

Minor remarks:

Figure 6 G-I: higher magnification images and separate color channels would be preferable.

REPLY: We have included higher magnification images of the immunofluorescence images in Fig. S5E, F with separate color channels for YFP-Ptch1, YFP-Smo, and acetylated-tubulin.

A number of data throughout the manuscript are indicated as "not shown", it would be good to show the data.

REPLY: We have included data that were not shown previously to Fig. S5A–D, G–I.

In the discussion, the authors state that "Here, we demonstrate that PAR3 is required for this appropriate transition by restricting proliferative NPC divisions in early telencephalic development". Not exactly: actually, what we are seeing is both increased and ectopic proliferation and we cannot judge whether or not this increase is a consequence of a possible apical detachment…

REPLY: To support our model, we inhibited hedgehog signaling *in vivo* and assessed the proliferation of NPCs (page 12, lines 377–379). Using cyclopamine, we found that the inhibition of Smo activity in the *Par3* cKO telencephalon resulted in a significant attenuation of NPC proliferation but not the ectopic distribution of NPCs (Fig. 7D–F). These data suggest that PAR3 is required for the restriction of proliferative NPC divisions by a mechanism dependent on restricting Smo activity (page 12, lines 387–389).

Discussion lines14-16

"Although we also examined the telencephalon of mice with conditional deletion of PAR3 with the Nestin-Cre transgene (NesCreTg+; Par3ΔE3/floxE3) in the early neurogenic phase around E11.5 (Fig. S2C,D) (Imai et 15 al., 2006), it exhibited much milder phenotypes than the Par3 cKO (Foxg1+/Cre; Par3ΔE3/floxE3) telencephalon (Fig. S2E-S2J)."

This sentence is very confusing: S2CD is first mentioned and does correspond to an early stage (E11.5) but S2E-J are E12.5-E14.5, and do not show an obvious phenotype while FoxG1Cre-Par3cKO show a strong phenotype from 11.5. The obvious interpretation is that Par3 KO is deleterious only when it takes place at early stage. This could be stated more explicitly, and the difference in expression onset between FoxG1 and Nestin could also be explained more explicitly.

REPLY: In accordance with this suggestion, we have improved the description to compare the different phenotypes observed in the *NesCre*$^{Tg+}$*;Par3*$^{\Delta E3/floxE3}$, and *Foxg1*$^{+/Cre}$*;Par3*$^{\Delta E3/floxE3}$ telencephalons and explained the difference from the aspect of onset between *NesCre* and *FoxG1Cre* more clearly (page 6, lines 179–183). We also indicated that PAR3 is required for the appropriate regulation of NPC proliferation within a narrow window of development (from line 184 on page 6 to line 186 on page 7).

Ectopic progenitors may result from a loss of planar spindle orientation (however the defects are modest and I find it unlikely that they would by themselves result in such a disorganization).

REPLY: We have improved the figure to clearly show the spindle orientation in the ventricular surface and the Brunner–Munzel test indicated a statistically significant difference between the *Par3* cKO and control telencephalons (Fig. 4E, F).

The Notch pathway target Hes5 is enriched, but further biochemical exploration of Notch pathway players fails to confirm a strong dysregulation of the pathway (and indeed, the enrichment in progenitors over neurons described earlier may suffice to explain the increase in Hes5 transcripts, although the authors do not propose this interpretation).

REPLY: We agree with this interpretation and have included it in the revised manuscript (page 10, lines 301–304).

Responses to Reviewer 2:

1) Par3 is inactivated in the dorsal as well as the ventral telencephalon. Neural stem cells in these two structures differ very much in their proliferation and differentiation characteristics but the authors do not seem to distinguish between these two regions. It rather appears that some analysis were performed in the ventral telencephalon, others with the dorsal telencephalon or even with whole telencephalic extracts. The manuscript would greatly benefit from a separate analysis of dorsal and ventral telencephalon. This is particularly important given the proposed role of increased Shh signalling. Whereas this pathway is active in the ventral telencephalon under physiological conditions, it is inactive in the dorsal telencephalon. Therefore, the model the authors propose that the expansion phase is characterized by high levels of Shh signalling does not apply to dorsal telencephalic progenitors.

REPLY: In accordance with this suggestion, we have indicated which telencephalic regions were analyzed and described the phenotypes in *Par3* cKO telencephalons in the dorsal and ventral regions as far as possible (Figs. 4A–C, 4H–J, 7A, B). To support our model that the restriction of Shh signaling is also required for the dorsal telencephalon, we have cited papers showing that this signaling is involved in the development of the dorsal cortex (Komada et al., 2008; Matsumoto et al., 2020; Wang et al., 2016) (page 13, lines 401–404).

2) An important caveat the authors need to address refers to a potential patterning defect caused by the enhanced Shh signalling. A ventralisation of dorsal progenitors could cause dramatic changes in their proliferation/differentiation characteristics. To this end, they need to carefully investigate the expression of ventral and dorsal progenitor and neuron markers in the Par3 mutant.

REPLY: We have assessed the dorsoventral patterning in the telencephalon by the immunofluorescent analysis of several marker proteins, including Shh, Nkx2.1, Gsx2/Gsh2, Ascl1 (ventral markers), Pax6, and Tbr2, (dorsal markers) (page 11, lines 322–327). This analysis revealed sharp pallial-subpallial boundaries in the *Par3* cKO telencephalons similar to the controls (Fig. S4), suggesting no severe defects had occurred in dorsoventral patterning.

3-1) While they note increased Gli1 expression, the expression of Ptch1, a commonly used marker of Shh signaling, is not altered. The authors do not comment on this difference.

REPLY: Although we did not detect a significantly increased accumulation of *Ptch1* mRNA in the *Par3* cKO telencephalon at E10.5, it was increased in neurospheres derived from *Par3* cKO embryos at E11.5 by a Smo-dependent mechanism (Fig. 5E). These observations suggest that the accumulation of *Ptch1* mRNA might be followed by increased *Gli1* mRNA expression (page 10, lines 314–317). This is consistent with the fact that Gli1 protein enhances *Ptch1* mRNA transcription.

3-2) They should also compare the Par3 phenotype with that of Gli3 conditional mutants.

REPLY: As mentioned in the response letter, it would be necessary to prepare mice on the 129SvJ background for tissue specific Cre expression from the *Foxg1Cre* allele (Hébert et al. Dev Biol. 2000). This major difficulty prevented us from trying this suggestion within the limited revision time available.

3-3) Moreover, primary cilia are not only involved in mediating the activation of Shh signalling but also in the formation of the Gli3 repressor. The authors need to analyse the levels of Gli3 activator and repressor forms and the ratio between the two forms in the dorsal and ventral telencephalon separately.

REPLY: For western blotting using anti-Gli3 pAb, we prepared telencephalons at E11.5 by separately dissecting them into dorsal and ventral parts. Although we found that the ratio between Gli3FL and Gli3R was higher in ventral than dorsal parts, no significant difference

was detected between Par3 cKO and control telencephalons in both parts (Fig. 5E, F) (page 10, lines 289–291). These data suggest that the processing of Gli3FL into Gli3R is not significantly affected in dorsal and ventral parts in the *Par3* cKO telencephalon at E11.5.

3-4) They should also discuss the role of primary cilia in telencephalic progenitors as revealed by several mouse mutants.

REPLY: On the basis of this suggestion, we have included a new discussion on the role of primary cilia in telencephalic progenitors (page 14, lines 427–436).

4) The two main findings of the manuscript, increased neural stem cell proliferation and increased Shh expression, are not linked by experiments. The authors need to perform either genetic or pharmacological rescue experiments. For example, they could cross Par3 mutants with Smo mutants or treat Par3 mutants with cyclopamine to inhibit Shh signaling.

REPLY: In accordance with this suggestion, we performed a pharmacological rescue experiment using cyclopamine (page 12, lines 377–379). We found the inhibition of Smo activity in the *Par3* cKO telencephalon resulted in the significant attenuation of NPC proliferation but not the ectopic distribution of NPCs (Fig. 7D–F). These data suggest that PAR3 is required for the restriction of proliferative NPC divisions in a manner dependent on restricting Smo activity (page 12, lines 387–389).

5) The identity of the progenitors in the ectopic SVZ needs to be clarified by staining for Tbr2 (intermediate progenitors) or for Hopx (outer radial glial cells). Alternatively, these cells could have required a ventral fate (see point 2). This could be investigated with Gsx2, Dlx2 or Ascl1 staining.

REPLY: In accordance with this suggestion, we performed immunostaining for Tbr2, Gsx2/Gsh2, Ascl1, and Pax6 (Fig. S4) as well as Sox2 and activated Notch intracellular domain (Fig. 3I, J). These data revealed that ectopic NPCs in the *Par3* cKO telencephalon had preserved self-renewal activity and differentiation potential at a level similar to those in other and control NPCs (page 7, lines 206–208). Unfortunately, we could not determine appropriate staining conditions using mouse anti-Hopx monoclonal antibody (IgG1, E-1, Santa Cruz #sc-398703, lot=I1021) in order to observe significant signals in the telencephalon at E11.5.

6) Many experiments are not or only inappropriately quantified. Experiments with lack of quantification:
Fig. 1D; Fig. 2; Fig. 4B, D; Fig. 5B. Figure 3D and F only use one embryo and two embryos, respectively. Therefore, n should be 1 and 2, respectively. The authors need to increase the number of analysed embryos to at least three and compare the averages from each embryo to avoid pseudo-replication. Statistical significance needs to be indicated for all experiments which, for example, is missing in Fig. 6C, H and J. Without this information, it is impossible to judge the outcome of these experiments.

REPLY: Following this indication, we added quantification information to Fig. 1D; Fig. 4B, E, F; and Fig. 5B. We also increased the number of analyzed embryos as far as possible (Fig. 3M, N; Fig. 4B, C; Fig. 6C) and included statistical analysis using the Brunner–Munzel test with sufficient quantification required for comparing differences (e.g. Fig. 6C, H and J). We also explicitly indicated the numbers of cells or cilia analyzed in the corresponding figures.

Second decision letter

MS ID#: DEVELOP/2021/199931

MS TITLE: PAR3 Restricts the Expansion of Neural Precursor Cells by Regulating Hedgehog Signaling

AUTHORS: Tomonori Hirose, Yoshinobu Sugitani, Hidetake Kurihara, Hiromi Kazama, Chiho Kusaka, Tetsuo Noda, Hidehisa Takahashi, and Shigeo Ohno

I have now received all the referees reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. The referees' suggest several minor changes that would improve clarity of the manuscript. In addition both referees indicate that treated and untreated Par cKO embryos should be compared with control embryos treated with vehicle.Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so. If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the referee's comments, and we will look over this and provide further guidance.

Reviewer 1

Advance summary and potential significance to field

In the revised version of the manuscript, Hirose and colleagues have addressed my main concerns from the first round of review:

- They have better characterized the disorganization of the VZ in Par3 cKO
- They have shown that the increased Smo accumulation that they had described upon Par3 knock-down in the 3T3 cell system is also observed in vivo (although to a much lower extent than in vitro), supporting their hypothesis that abnormal cilium organization in the absence of Par3 facilitates Smo accumulation in the (abnormal) cilium, and therefore possibly explaining the increased Shh signaling activity that results in the modest delay in neurogenesis that they observe in the ventral telencephalon at E10.5.
- Finally, they have made other changes to the text that render the manuscript easier to read and follow.

I have no main concerns remaining with the paper

Comments for the author

Minor comment:

In response to comments from reviewer#2, the authors have added a "rescue" experiment with cyclopamin, which does not restore at all the structural defects caused by the early loss of Par3 (indicating that these defects are independent from Shh signaling), but reduces the number of PH3+ cells observed at E10.5 in the Par3 cKO. Although this result aligns well with the proposed scenario, one should be cautious with its interpretation: I expected that cyclopamin would also reduce the number of PH3+ cells in control embryos, but this experiment is not provided. Even if it were, it would be difficult to predict whether the effect for a given dose of cyclopamin should be more intense in Par3 cKO than in control. Any treatment that inhibits proliferation in the wt (even independently of Shh) would also probably do it in the Par3 mutant, so although cyclopamin counteracts the Par3 phenotype, I do not think one can definitely conclude that it results from a direct rescue at the molecular level. I therefore agree with the authors when they write in the last paragraph of the Result section that "these observations support the notion that the Smo-dependent hyperactivation of Hh signaling increases NPC proliferation in the Par3 cKO telencephalon", but I would suggest to slightly tone down the last two sentences of the introduction (p4, lines 114-116), as the results suggest, rather than formally demonstrate, that the loss of Par3 leads to hyperproliferation via hyperactivation of Hh signaling.

Page 4, line 101: the word "defects" is missing in the sentence after "ciliary"

Page12, line 369: "(Fig. S5E-G)": I think the authors refer to fig. S5F-I

Reviewer 2

Advance summary and potential significance to field

In their manuscript, Hirose et al., investigate the molecular mechanisms that underlie the switch from symmetric proliferative to asymmetric neurogenic divisions in neural stem cells. To this end, they generated and characterized Par3 conditional mouse mutants in which Par3 is specifically inactivated in the telencephalon at the time this switch occurs. They found that the Par3 mutation leads to increased neural progenitor proliferation and the formation of an expanded subventricular zone. On a cellular level, these changes coincided with alterations in interkinetic nuclear migration, in the orientation of the mitotic spindle and most notably in the morphology of the primary cilium. Consistent with ciliary defects, they also detected increased Shh signalling and showed increased Smo ciliary localisation in 3T3 Par3 knock-down cells.

Investigating the mechanisms they lie at the basis of neural stem cells to switch to neurogenic cell divisions is of great importance as it determines the size of the neural stem cell pool and ultimately the size of the developing brain. Alterations in this switch are at the centre of the evolutionary expansion of the human brain and of several neurodevelopmental disorders such as microcephaly and macrocephaly. On the other hand, very little is known how this crucial developmental step is controlled. Hence, this manuscript addresses an important, open question in Neurodevelopmental Biology and I recommend the publication of this manuscript, however, there are a number of major points which need to be addressed first.

Comments for the author

The authors addressed most of my concerns, but there are still some issues that require careful consideration.

The western blot in Fig. 1D provides some numbers, but lacks a statistical analysis.

The authors quantified the Gli3FL/Gli3R ratio, but they should also quantify the levels of Gli3FL and Gli3R, especially as cortical stem cell development depends on Gli3R levels and not on the Gli3FL/Gli3R ratio.

The authors conclude that a larger proportion of Par cKO NPCs maintained self-renewal activity in the telencephalon, but this change is statistically significant only for ventral, but not for dorsal NPCs. Hence, they need to be careful not to overstate their results and ensure throughout the manuscript that they observed a spatially restricted effect on self-renewal. They cannot conclude that conditional inactivation of Par3 affects the switch from symmetric proliferative to asymmetric divisions throughout the telencephalon.

I did not ask for a rescue experiment in my first review, but I noted that this analysis is not performed properly. The authors need to compare treated and untreated Par cKO embryos with control embryos treated with vehicle. Furthermore, they only analyse the number of mitotic cells and their distribution but do not investigate self-renewal vs. asymmetric cell division, a major finding of their study.

Second revision

Author response to reviewers' comments

Responses to Reviewer 1:

1) In response to comments from reviewer#2, the authors have added a "rescue" experiment with cyclopamine, which does not restore at all the structural defects caused by the early loss of Par3 (indicating that these defects are independent from Shh signaling), but reduces the number of PH3+ cells observed at E10.5 in the Par3 cKO. Although this result

aligns well with the proposed scenario, one should be cautious with its interpretation: I expected that cyclopamine would also reduce the number of PH3+ cells in control embryos, but this experiment is not provided. Even if it were, it would be difficult to predict whether the effect for a given dose of cyclopamine should be more intense in Par3 cKO than in control. Any treatment that inhibits proliferation in the wt (even independently of Shh) would also probably do it in the Par3 mutant, so although cyclopamine counteracts the Par3 phenotype, I do not think one can definitely conclude that it results from a direct rescue at the molecular level. I therefore agree with the authors when they write in the last paragraph of the Result section that "these observations support the notion that the Smo-dependent hyperactivation of Hh signaling increases NPC proliferation in the Par3 cKO telencephalon", but I would suggest to slightly tone down the last two sentences of the introduction (p4, lines 114-116), as the results suggest, rather than formally demonstrate, that the loss of Par3 leads to hyperproliferation via hyperactivation of Hh signaling.
REPLY: We agree with Reviewer 1 and recognize the limitations of such epistatic experiments that do not provide definitive conclusions at the molecular level. Accordingly, we have toned down the statements to suggest that PAR3 restricts NPC expansion via the regulation of Hh signaling (page 2, lines 45–48; page 4, lines 113–116).

2) *Page 4, line 101: the word "defects" is missing in the sentence after "ciliary"*
REPLY: We corrected this point in the revised manuscript (page 4, line 99).

3) *Page12, line 369: "(Fig. S5E-G)": I think the authors refer to fig. S5F-I*
REPLY: As suggested, we corrected all the text citing Fig. S5E–I in the revised manuscript (page 11, line 352; page 12, lines 359, 360, and 363).

Responses to Reviewer 2:

1) *The western blot in Fig. 1D provides some numbers, but lacks a statistical analysis.*
REPLY: In response, we have performed statistical analysis using the two-tailed Welch's *t*-test to show the significant loss of PAR3 protein in the *Par3* cKO telencephalon (page 5, line 139).

2) *The authors quantified the Gli3FL/Gli3R ratio, but they should also quantify the levels of Gli3FL and Gli3R, especially as cortical stem cell development depends on Gli3R levels and not on the Gli3FL/Gli3R ratio.*
REPLY: Based on the results shown in Fig. 5E, we provided bar graphs to show the levels of Gli3FL and Gli3R normalized to Sox9, respectively (Fig. S3C, D). The two-tailed Welch's *t*-test showed there was no significant difference in either Gli3 isoform between the *Par3* cKO and control telencephalons in the dorsal and ventral regions.

3) *The authors conclude that a larger proportion of Par3 cKO NPCs maintained self-renewal activity in the telencephalon, but this change is statistically significant only for ventral, but not for dorsal NPCs. Hence, they need to be careful not to overstate their results and ensure throughout the manuscript that they observed a spatially restricted effect on self-renewal. They cannot conclude that conditional inactivation of Par3 affects the switch from symmetric proliferative to asymmetric divisions throughout the telencephalon.*
REPLY: In accordance with this comment, we tried to not overstate our results and we have toned down some of our language as suggested (page 2, lines 45–48; page 9, lines 263–265; page 13, lines 390–391; page 15, lines 452–454).

4) *The authors need to compare treated and untreated Par3 cKO embryos with control embryos treated with vehicle. Furthermore, they only analyse the number of mitotic cells and their distribution but do not investigate self-renewal vs. asymmetric cell division, a major finding of their study.*

REPLY: We agree with Reviewer 2 on the first point. We compared the proliferation and distribution of NPCs in the treated and untreated *Par3* cKO embryos with control embryos treated with vehicle (page 12, lines 376–381; Fig. 7D–F). With regard to the second point, we understand it would be ideal to analyze self-renewal vs. asymmetric cell division in these embryos treated with cyclopamine or vehicle. However, it was difficult for us to accept this suggestion because of the following reasons.

1. The journal deadline for resubmission meant there was insufficient time to prepare the necessary number of embryos for these experiments and to analyze all the indicated phenotypes.
   In the first review, it was suggested that pharmacological rescue experiments with cyclopamine might help link the increase in neural precursor cell proliferation and hyperactivation of hedgehog signaling in *Par3* cKO embryos. Thus, we focused on the proliferation of NPCs in our "rescue" experiment within the limited time provided for revision. However, because the analysis of self- renewal vs. asymmetric cell division was not mentioned in the first review, we did not prepare samples to analyze this aspect in the experiments for our first revision.

2. The limitations of epistatic experiments made us hesitate to expand the analysis further.
   As Reviewer 1 commented, such epistatic experiments do not provide definitive conclusions at the molecular level. Considering these potential caveats in the "rescue" experiment, we toned down the language suggesting the loss of Par3 leads to hyperproliferation via the hyperactivation of Hh signaling (page 2, lines 45–48; page 4, lines 113–116).

Third decision letter

MS ID#: DEVELOP/2021/199931

MS TITLE: PAR3 Restricts the Expansion of Neural Precursor Cells by Regulating Hedgehog Signaling

AUTHORS: Tomonori Hirose, Yoshinobu Sugitani, Hidetake Kurihara, Hiromi Kazama, Chiho Kusaka, Tetsuo Noda, Hidehisa Takahashi, and Shigeo Ohno
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

The authors have addressed my main remarks.

Comments for the author

no more suggestions

Reviewer 2

Advance summary and potential significance to field

In their manuscript, Hirose et al., investigate the molecular mechanisms that underlie the switch from symmetric proliferative to asymmetric neurogenic divisions in neural stem cells. To this end,

they generated and characterized Par3 conditional mouse mutants in which Par3 is specifically inactivated in the telencephalon at the time this switch occurs. They found that the Par3 mutation leads to increased neural progenitor proliferation and the formation of an expanded subventricular zone. On a cellular level, these changes coincided with alterations in interkinetic nuclear migration, in the orientation of the mitotic spindle and most notably in the morphology of the primary cilium. Consistent with ciliary defects, they also detected increased Shh signalling and showed increased Smo ciliary localisation in 3T3 Par3 knock-down cells.

Investigating the mechanisms they lie at the basis of neural stem cells to switch to neurogenic cell divisions is of great importance as it determines the size of the neural stem cell pool and ultimately the size of the developing brain. Alterations in this switch are at the centre of the evolutionary expansion of the human brain and of several neurodevelopmental disorders such as microcephaly and macrocephaly. On the other hand, very little is known how this crucial developmental step is controlled. Hence, this manuscript addresses an important, open question in Neurodevelopmental Biology.

Comments for the author

The authors addressed my remaining concerns, it would just be nice if they could provide a diagram illustrating the statistical test of the Par3 Western blot (Figure 1D).