CAMSAP3 is required for mTORC1-dependent ependymal cell growth and lateral ventricle shaping in mouse brains

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AUTHORS: Toshiya Kimura, Hiroko Saito, Miwa Kawsaki, and Masatoshi Takeichi

I have now received the reports of three referees on your manuscript and I 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. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy 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 currently be unable to access the lab to undertake experimental revisions. 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 paper of Kimura et al. "CAMSAP3 is required for mTORC1-dependent ependymal cell growth and lateral ventricle shaping in mouse brains" describes that CAMSAP3, while mediating non-centrosomal microtubule assembly, is required for mTORC1-dependent maturation of ependymal cells at the neocortex of developing mouse brains and the loss of CAMSAP3 causes deformation of the lateral ventricles. The abnormally narrow lateral ventricles form in Camsap3 mutant mice, where excessive stenosis or fusion was induced causing a decrease of neural stem cells at the ventricular and subventricular zones.

Results: page 10, "apical domain growth in CAMSAP3-mutated ependymal cells was impaired during postnatal periods", and (page 11) "only the non-ciliated areas failed to grow normally in the mutants". This is a major finding explaining the reduction of the ventricular cavity.
page 16, "CAMSAP3 produces apical most MT networks in ependymal cells and the lack of functional CAMSAP3 causes their depletion". This is useful novel information.
The figures nicely organized with good quality microscopy and various formats of data (primary ones, graphs and cartoons) well-integrated to illustrate the points of the paper.

Comments for the author

page 15, Figure 6 shows ChP, which is mentioned in the Figure legend only as abbreviation and not mentioned in the text at all. It has been suggested that cilia are less common in ependyma covering the circumventricular organs (CVOs; Krisch B, Leonhardt H, Buchheim W (1978) The functional and structural border between the CSF-and blood-milieu in the circumventricular organs (organum vasculosum laminae terminalis, subfornical organ, area postrema) of the rat. Cell Tissue Res 195:485-97. doi: 10.1007/BF00233891; Gross PM, Weindl A (1987) Peering through the windows of the brain. J. Cereb. Blood Flow Metab. 7:663-72. DOI: 10.1038/jcbfm.1987.120). ChP belonging to CVOs is debatable. Perhaps, the authors may evaluate critically their own relevant data and extend their discussion by commenting on connection between CAMSAP3, cilia and ependyma of CVOs in the context of distribution of ciliated cells in different regions of ependyma.
page 16, the CAMSAP3–non-centrosomal MT system is important for morphogenesis of the lateral ventricles.
Loss of it brought about narrowing as well as increased stenosis or fusion of the ventricles; ibid, "the lysosomal mispositioning in mutant cells might have been a cause of mTORC1 activity reduction".
Since cell survival is not affected, perhaps authors may discuss an importance of lysosomal mispositioning?

Minor comments:
CAMSAP3 defines the human protein; for the murine protein, Camsap3 name will be more appropriate; page 15, para 1, line 3, and second line from below, the inconsistent usage of abbreviation BB, with "basal bodies" used in the text after the abbreviation BB was introduced.

Reviewer 2

Advance summary and potential significance to field

Takeishi and colleagues address an important topic, namely how loss of proteins associated with microtubule impact brain functions, such as neurogenesis. As many papers so far deal with microtubule modifications at the cellular level only, this report goes to function. The authors provide experiments figuring out the mechanism behind Camsap3 loss which occurs in ependymal cells.

Comments for the author

Basic statements in introduction should be referenced in more detail. For example, the first paragraph of the introduction has only 2 citations, but there are some fundamental statements referring to previous literature not cited.

Figure organization could be improved so that the reader could orientate better, e.g. Figure 1 WT and mutatnt is written on top of panels in A, while it is stated vertically orientated in D. Sometimes it is C3 mutant but also Camsap3 mutant.
Camsap 3 and S100beta immunostaining, a key point of the paper: a higher magnification is needed to visualize the immunostaining on the cellular basis. See also Figure 2, BrdU colocalization or Vcam1/GFAP immunostaining. See also Figure S6D.

NSC generate different types of olfactory bulb interneurons, such as granule cells and periglomerular cells, which are biochemically very diverse. Is there a general loss or loss of a specific neuron type, hence a loss of a specific stem cell type?

Consider repeating key findings with conditional deletion of Camsap 3.

The evidence that embryonic development occurs normally, but adult neurogenesis only is affected has to be corroborated. What about the overall cellular architecture in mutant mice?

Is the cellular architecture of the remaining NSC normal? Is their potential reduced or are they only less proliferating?

Are microglial activated due to the stenosis?

While the mechanism in ependymal cells is worked out very well, the effects of camsap3 mutation on NSCs is less clear.

Data on the changes of the microtubular network in ependymal cells on the light microscopic level are not entirely convincing. Especially as the images are really small. Consider electron microscopy or at least some super resolution technique to corroborate this key finding.

Reviewer 3

Advance summary and potential significance to field

In the manuscript by Kimura et al, the authors describe a brain ventricular phenotype in a mouse knock-out model lacking the protein CAMSAP3. The authors studied the molecular mechanism leading to the reduced brain ventricles and the impact of this phenotype on brain development. The authors identified that defective microtubules, results in lysosomal rearrangements and reduced mTORC1, thus leading to defective ependymal cell growth. In general the manuscript is well written, of broad interest, supported by well performed and controlled experiments. Thus, I favor the publication of this article.

Comments for the author

I recommend the authors to review the points indicated below to improve their manuscript and the scope of their work.
1. Actin meshwork has been shown in multiciliated cells to anchor the BB and protect them from shear forces. How does the actin meshwork in ependymal cells relate to the microtubules network. It would be good if the authors could clarify this in their text.
2. The cytoskeleton is also affected by the planar polarity genes (PCP). The authors may want to include some clarification on the meaning of their results in the context of cell polarity.
3. The manuscript would benefit from further discussion on the lack of hydrocephalus in the mutant mice as the lack of hydrocephalus is a surprising result given the stenosis and fusion on the ventricle.
4. In the analysis of the olfactory bulb, the authors claim that the reduced size is due to a decrease of adult NSC. Is it possible that the rostro-migratory stream is also affected due to the stenosis/fusion of the ventricle? It would be good if the authors could clarify this.
5. The authors stain CAMSAP3 in CAMSAP3 knock-out animals. It is not very clear to me why the author performed this experiment. Is the antibody recognizing the non mutated part of the protein? Do the author expect a certain impact of the mutation on the localization of the protein? It would be good if the authors could clarify this.

6. Figure 5E-F, it may help the readers to have an overlay of the p-Akt and ZO1 signal.
7. The connection between p-Akt and mTORC is not very clear in the result section. The authors may want to clarify the meaning of those results and correlate these two findings better in their result section.
8. The authors used tyr-tubulin in Fig6C to label the microtubules, but do not discuss why they used this modification.

First revision

Author response to reviewers' comments

We are thankful to all three reviewers, who have carefully evaluated our work. Our responses to each comment (in italic) are explained below. In the revised text, major changes made in response to reviewer feedback are shown in blue font. Figures have also been updated to incorporate these changes.

Reviewer 1

page 15, Figure 6 shows ChP, which is mentioned in the Figure legend only as abbreviation and not mentioned in the text at all. It has been suggested that cilia are less common in ependyma covering the CVcircumventricular organs (CVOs; Krisch B, Leonhardt H, Buchheim W (1978) The functional and structural border between the CSF-and blood-milieu in the circumventricular organs (organum vasculosum laminae terminalis, subfornical organ, area postrema) of the rat. Cell Tissue Res 195:485-97. doi: 10.1007/BF00233891; Gross PM, Weindl A (1987) Peering through the windows of the brain. J. Cereb. Blood Flow Metab. 7:663-72. DOI: 10.1038/jcbfm.1987.120). ChP belonging to CVOs is debatable. Perhaps, the authors may evaluate critically their own relevant data and extend their discussion by commenting on connection between CAMSAP3, cilia and ependyma of CVOs in the context of distribution of ciliated cells in different regions of ependyma.

Response: We are grateful to the reviewer for pointing out this important issue. We reconfirmed through the literature that ependymal cells adjacent to the choroid plexus (ChP) have less cilia, forming CVOs. The image shown in Fig. 6F indeed includes part of ChP. However, after re-examining our data, we confirmed that ependymal cells in the image are not the ones contiguous to ChP. ChP arises from the ventral portion of the ventricle, reaching close to the cortex. Thus, a distal portion of ChP happened to be included in the section used to prepare Fig. 6F. In this work, we have concentrated our attention on the authentic population of ependymal cells for simplicity, and would like to leave the problem related to CVOs for future studies. To address the concern as raised by the reviewer, we changed the Fig 6F legend to read 'the choroid plexus and ependymal layer in the image are not structurally continuous'.

page 16, the CAMSAP3-non-centrosomal MT system is important for morphogenesis of the lateral ventricles. Loss of it brought about narrowing as well as increased stenosis or fusion of the ventricles; ibid, "the lysosomal mispositioning in mutant cells might have been a cause of mTORC1 activity reduction". Since cell survival is not affected, perhaps authors may discuss an importance of lysosomal mispositioning?

Response: On page 19, we rewrote the sentence including "the lysosomal mispositioning in mutant cells might have been a cause of mTORC1 activity reduction" to better explain our view:

"the lysosomal mispositioning observed in mutant cells ought to have reduced mTORC1 that is accessible to Akt, resulting in the lesser activation of mTORC1"

Minor comments:

CAMSAP3 defines the human protein; for the murine protein, Camsap3 name will be more appropriate;

Response: According to the 'International Protein Nomenclature Guidelines' https://www.ncbi.nlm.nih.gov/genome/doc/internatprot_nomenguide/, we should use an all uppercase gene symbol in a protein name for vertebrates. On the other hand, mouse gene symbols should begin with an uppercase letter followed by all lowercase letters, according to Mouse Nomenclature Home Page http://www.informatics.jax.org/mgihome/nomen/. We follow these guidelines and therefore use CAMSAP3 as the protein name, and *Camsap3*, for its gene. The paper that first proposed the name of this protein family also used 'CAMSAP' (see below):

Baines et al. (2009). The CKK domain (DUF1781) binds microtubules and defines the **CAMSAP**/ssp4 family of animal proteins. Mol Biol Evol. 26:2005-14. doi: 10.1093/molbev/msp115.

If our understanding of this issue is incorrect, we will be pleased to follow the reviewer's suggestion when we prepare a final version of the manuscript.

page 15, para 1, line 3, and second line from below, the inconsistent usage of abbreviation BB, with "basal bodies" used in the text after the abbreviation BB was introduced.

Response: We have now corrected the inconsistent usage of BB and basal bodies.

Reviewer 2

Basic statements in introduction should be referenced in more detail. For example, the first paragraph of the introduction has only 2 citations, but there are some fundamental statements referring to previous literature not cited.

Response: We have added more references to the revised manuscript, particularly at the first paragraph of the introduction.

Figure organization could be improved so that the reader could orientate better, e.g. Figure 1 WT and mutant is written on top of panels in A, while it is stated vertically orientated in D. Sometimes it is C3 mutant but also Camsap3 mutant.

Response: Thanks for this comment. We have changed the orientation of some panels (particularly in Figs. 1, 2 and 6) and also labels to improve the figures. Please check updated figures.

Camsap 3 and S100beta immunostaining, a key point of the paper: a higher magnification is needed to visualize the immunostaining on the cellular basis.

Response: As recommended, we added higher magnification views of CAMSAP3 and S100□ immunostain to Fig. 1G and H.

See also Figure 2, BrdU colocalization or Vcam1/GFAP immunostaining. See also Figure S6D.

Response: To respond this comment, we enlarged the images for BrdU/S100β immunostaining, placing them in Fig. 2K. Concerning VCAM/GFAP immunostaining, we prepared new images for Fig. S3A. In addition, we also enlarged the Fig. S6D images, re-orienting their panels for better visualization.

NSC generate different types of olfactory bulb interneurons, such as granule cells and periglomerular cells, which are biochemically very diverse. Is there a general loss or loss of a specific neuron type, hence a loss of a specific stem cell type?

Response: To respond to this comment, we have performed an additional experiment to test whether there is a subtype specificity in the loss of interneuron generation. We checked four major subtypes of interneurons: CalR$^+$ granule cells (GCs), calretinin (CalR)$^+$ periglomerular cells (PGCs), calbindin (CalB)$^+$ PGCs and tyrosine hydroxylase (TH)$^+$ PGCs. We counted the number of cells expressing one of these markers, which are co-labeled with BrdU, finding that newly generated (BrdU$^+$) neurons decreased in the CalR$^+$ GCs, whereas other subtypes did not decrease in a

statistically significant manner. Thus, there seems to be a subtype specificity in the loss of interneurons in CAMSAP3 mutant OBs (see the new Fig. S4H-N). We consider this result to be consistent with the observations of where the ventricle abnormality and the associated loss of adult neural stem cells took place. CalR$^+$ GCs are generated from stem cells present along the medial wall of the anterior portion of the lateral ventricle (Merkle et al., 2007. Science 317: 381-384), whose niche was severely disrupted in *Camsap3* mutant brains. We have added sentences describing these results on page 9.

Consider repeating key findings with conditional deletion of Camsap 3.

Response: Thanks for this important idea. Such experiments, if done, should be beneficial to confirm our conclusions. However, these will require a lengthy time for completion. Considering various factors in the current research environment, we would like to avoid extra tasks that are not absolutely necessary for publication of this work. We hope the reviewer agrees with this decision.

The evidence that embryonic development occurs normally, but adult neurogenesis only is affected has to be corroborated. What about the overall cellular architecture in mutant mice?

Response: As far as ependymal cells are concerned, their maturation occurs postnatally, as reported (Spassky et al. 2005. Journal of Neuroscience 25:10-18), and CAMSAP3 dysfunction appears to have affected the postnatal events only, as we did not find any detectable abnormality in their precursor cells at embryonic stages, as mentioned in the manuscript. As for striatum in CAMSAP3 mutants, we found no change in cell proliferation at embryonic stages. This negative data has now been added to Fig. S1D-F, as well as to the main text on page 8.

Regarding other cell types, such as epithelial tissues, we found major defects only at postnatal stages, as some examples were reported (Toya et al., 2015, PNAS 113: 332- 337; Robinson et al. 2020, PNAS 117: 13571-13579). On the other hand, we might have missed minor defects behind the normal-looking appearance of mutant tissues, as it was practically difficult to thoroughly check all the cells in the mutant embryo unless visible phenotypes were detectable. We therefore do not particularly claim that their embryonic development occurs normally. We re-read the manuscript and corrected places where we found overstatement regarding the reviewer's points.

Is the cellular architecture of the remaining NSC normal? Is their potential reduced or are they only less proliferating?

Response: As shown in Fig. S3B (originally S3A), we noted small changes in the shape of NSCs in CAMSAP3 mutants. To further analyze their morphology, we repeated immunostaining for GFAP/VCAM in NSCs, but did not find particular morphological differences between those in wild-type and *Camsap3*-mutated brains, as mentioned on page 9 and shown in new Fig. S3A, suggesting that CAMSAP3 dysfunction leads to only minor changes in NSC architecture. Concerning their proliferation, it was difficult to examine cell proliferation ability within the short span of experimental time that is given for revision of the manuscript. To determine neurogenic potential or proliferation of adult NSCs, clonal analysis or barcode labeling would be suitable, but both of these experiments are technically difficult and require considerable time. AraC treatment might be another option to clarify this concern, but, unfortunately, we have no experience to infuse drugs into the brain ventricle by means of cannula and osmotic pump. As the main focus of this study is to clarify how the ependymal cell layer lost normal architecture in *Camsap3* mutants, we would like to leave detailed analysis of NSC proliferation for future studies.

Are microglial activated due to the stenosis?

Response: To address this question, we examined activated microglia by immunostaining, finding no signs of their ectopic activation at around the areas of ventricular stenosis nor particular difference in their distribution between wild-type and *Camsap3*-mutated specimens (please see the data shown below). Since these data are not particularly related to the main story of our study, we did not add them to the manuscript.

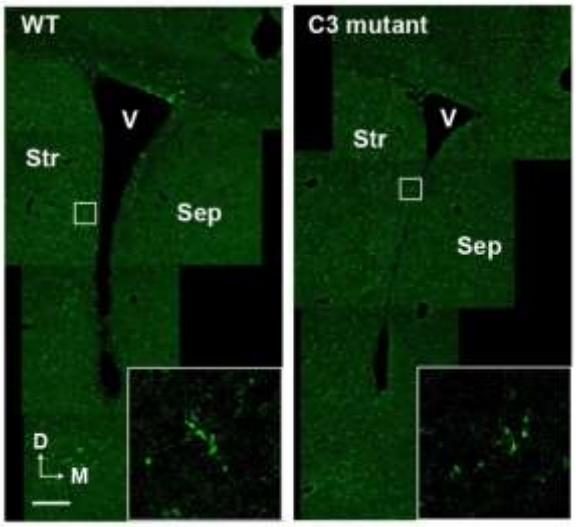

Figure legend. Coronal sections of WT or *Camsap3* mutant telencephalon at 28.5, immunostained for CD68, a marker for activated microglia. The squared region is enlarged in each panel. No change in CD68 immunosignals is observed in the mutant samples.
Abbreviations are identical to those in Fig. 2C.

While the mechanism in ependymal cells is worked out very well, the effects of camsap3 mutation on NSCs is less clear.

Response: Our observations suggest that the reduction of NSCs in mutants was mainly due to stenosis or fusion of the ventricle, although they exhibited small morphological changes in CAMSAP3 mutants as explained above. Therefore, we paid less attention to their cellular architecture. Our major focus in this study is on the problem of how the ependymal cell layer lost normal architecture in CAMSAP3 mutants, in order to understand anatomical mechanisms that caused stenosis or fusion of the ventricle.
Deeper cell level analysis of NSCs in CAMSAP3 mutants is intriguing, but beyond the scope of the current study.

Data on the changes of the microtubular network in ependymal cells on the light microscopic level are not entirely convincing. Especially as the images are really small. Consider electron microscopy or at least some super resolution technique to corroborate this key finding.

Response: Thanks for this comment. To improve the visibility of our images, we reorganized panels in Figure 6F, and modified other parts of the figure. Concerning the size of images, we used the maximum allowed page area (210 x 180 cm) to compile them.

To collect the images presented in this paper, we used Airyscan microscopy, whose resolution is often comparable to that of super-resolution microscopic images.
Regarding electron microscopy (EM), we actually used it for analysis of microtubules in ciliated cells of other organs such as trachea and oviducts (manuscript in preparation).
However, we noticed that it is difficult to capture the overall feature of microtubule organization using EM, because the magnification is too large, and sections are too thin. We therefore think that Airyscan microscopy is the best choice to describe microtubule distribution in our experiments.

Reviewer 3

1. Actin meshwork has been shown in multiciliated cells to anchor the BB and protect them from shear forces. How does the actin meshwork in ependymal cells relate to the microtubules network. It would be good if the authors could clarify this in their text.

Response: In response to this comment, we observed actin filaments at the apical regions of P7.5

ependymal cells by co-staining for □-tubulin (as a basal body marker) and F-actin (with phalloidin). We detected actin networks that are distributed around the BB cluster, as reported previously (e.g., Mahuzier et al., 2018). However, we did not find any difference in their distribution pattern between WT and mutant cells. We added these results to page 17 of the revised text and the data are shown in Fig. S7.

The cytoskeleton is also affected by the planar polarity genes (PCP). The authors may want to include some clarification on the meaning of their results in the context of cell polarity.

Response: To respond to this comment, we have analyzed PCP of ependymal cells using samples in which N-cadherin (as a junctional marker) and □-tubulin (as a basal body marker) were immunostained. The results showed that 'translational polarity' at least was not affected by CAMSAP3 dysfunction. This finding is mentioned on page 17 of the revised manuscript, and shown in new Fig. S6E and S6F. We also inferred that 'rotational polarity' is not affected, because cilia in *Camsap3*-mutated ependymal cells beat normally (see below).

The manuscript would benefit from further discussion on the lack of hydrocephalus in the mutant mice, as the lack of hydrocephalus is a surprising result given the stenosis and fusion on the ventricle.

Response: One of the major causes of hydrocephalus is defects in cilia motility. We therefore examined the movement of cilia in *Camsap*3-mutated ependymal cells by live imaging, and found that they are beating normally, unlike the results obtained using nasal epithelial cilia (Robinson et al. 2020, PNAS 117: 13571-13579). We added this finding to the manuscript as Movies 1 and 2, mentioning it on pages 17 and 19. We also discussed why *Camsap*3 mutant brains did not show hydrocephalus on page 20.

In the analysis of the olfactory bulb, the authors claim that the reduced size is due to a decrease of adult NSC. Is it possible that the rostro-migratory stream is also affected due to stenosis/fusion of the ventricle? It would be good if the authors could clarify this.

Response: We appreciate this comment. We have attempted to address this possibility by tracing migrating cells in sagittal sections of adult brains, but failed to obtain convincing results to date. Therefore, we want to leave this subject for future studies. Since the main focus of this work is to reveal how ependymal layers are affected by CAMSAP3 dysfunction, we believe that the absence of data on neuroblast migration does not affect the main conclusion of the manuscript.

The authors stain CAMSAP3 in CAMSAP3 knock-out animals. It is not very clear to me why the author performed this experiment. Is the antibody recognizing the non mutated part of the protein? Do the author expect a certain impact of the mutation on the localization of the protein? It would be good if the authors could clarify this.

Response: Our antibody for CAMSAP3 recognizes the mutant (truncated) CAMSAP3 protein expressed in *Camsap3*$^{dc/dc}$ mice, as described in our previous report (Toya et al., 2015, PNAS 113: 332-337, https://doi.org/10.1073/pnas.1520638113). We presented the data of the truncated CAMSAP3 molecules just as basic information on where they are localized in the *Camsap3* mutant brains. We believe that the immunosignals for the truncated CAMSAP3 are useful for confirming the relative position of CAMSAP3 to other molecules when readers compare wild-type and mutant cells.

6. Figure 5E-F, it may help the readers to have an overlay of the p-Akt and ZO1 signal.

Response: We appreciate this recommendation. We prepared an overlay of p-Akt and N-cadherin rather than the p-Akt and ZO1 signal, and placed it in the lower row of Fig. 5E, as the anti-N-cadherin antibody used for this experiment (mAb MNCD2) most intensely reacted with the N-cadherin localized along the adherens junctions, giving an image similar to that of ZO1. This binding characteristic of mAb MNCD2 is also mentioned in the legend of Fig. 5E.

The connection between p-Akt and mTORC is not very clear in the result section. The authors may want to clarify the meaning of those results and correlate these two findings better in their result section.

Response: We rewrote the sentences to relate the observations of p-Akt to the mTORC experiments. See the last paragraph of the section 'Reduction of lysosomes at apical regions of Camsap3 mutant ependymal cells' (pages 14 and 15).

The authors used tyr-tubulin in Fig6C to label the microtubules, but do not discuss why they used this modification.

Response: We have added sentences explaining why we used tyrosinated tubulin to examine microtubules to the revised text on page 16. Briefly, antibodies for ¤-tubulin too strongly labeled ciliary microtubules, which hindered our observations of the cytoplasmic microtubules in ependymal cells. By contrast, antibodies for 'tyrosinated' tubulin gave only moderate immunostaining signals on the ciliary microtubules, allowing us to closely observe microtubules at the apical-most cytoplasm of the cell.

Second decision letter

MS ID#: DEVELOP/2020/195073

MS TITLE: CAMSAP3 is required for mTORC1-dependent ependymal cell growth and lateral ventricle shaping in mouse brains

AUTHORS: Toshiya Kimura, Hiroko Saito, Miwa Kawsaki, and Masatoshi Takeichi
ARTICLE TYPE: Research Article

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

Reviewer 2

Advance summary and potential significance to field

This paper highlights the connection of a cytoskeletal regulator (CAMSAP3) to cellular functions and architecture of ependymal cells.

Comments for the author

The authors have added additional new data addressing my concerns. The overall composition of the figures and images is now clear.
I think this is very nice paper that provides new conceptual insights into the mechanisms of how Camsap3 regulates microtubules and in turn influences overall ependymal architecture and adult neurogenesis.