JCB

CAMSAPs organize an acentrosomal microtubule network from basal varicosities in radial glial cells

Laure Coquand, Guiliana Victoria, Alice Tata, Jacopo Carpentieri, Jean-Baptiste Brault, Fabien Guimiot, Vincent Fraisier, and Alexandre Baffet

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Re: JCB manuscript #202003151

Dr. Alexandre D Baffet
Institut Curie, PSL Research University, CNRS UMR144, Paris, France
12, rue Lhomond
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France

Dear Dr. Baffet,

Thank you for submitting your manuscript entitled "A dendritic-like microtubule network is organized from basal fiber swellings in neural progenitors". We apologize for the delay in providing you with a decision. n any case, the manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

You will see that although all three reviewers have voiced enthusiasm for the study, they each raise a number of overlapping concerns which will need to be addressed before the paper would be suitable for publication in JCB.
The reviewers have noted the relatively descriptive nature of the current study and have suggested that further mechanistic insight may be necessary. Provided that the paper remains a Report, we do not feel that full mechanistic elucidation will be necessary for the revision. However, we agree with reviewers #1 and #3 that further 'causative' data such as knockdown of CAMSAP would be essential. In addition, we feel that examination of the endogenous proteins (as noted by reviewer #2, pt#5) must also be addressed in the revision. We hope that you will be able to fully address these and each of the other reviewer concerns in a revised manuscript.

As you may know, the typical timeframe for revisions is three to four months. However, we at JCB realize that the implementation of social distancing and shelter in place measures that limit spread of COVID-19 also pose challenges to scientific researchers. Lab closures especially are preventing scientists from conducting experiments to further their research. Therefore, JCB has waived the revision time limit. We recommend that you reach out to the editors once your lab has reopened to decide on an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

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When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Erika Holzbaur, PhD
Monitoring Editor
Journal of Cell Biology

Tim Spencer, PhD
Executive Editor
Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

In this study the authors characterize the microtubule cytoskeleton in polarized radial glia. Looking at basal and apical fibers of these cells by EB3 imaging, they found that there was a difference in the polarity of microtubules in the apical and basal fibers, which was previously unknown. Specifically, microtubules in the apical fibers are uniformly polarized with minus ends directed towards the ventricular side, but they are of mixed polarity in the basal fibers. Furthermore, they found that focal varicosities in the basal fibers contained components that may lead to extra centrosomal nucleation of microtubules. All of this is shown in situ using an imaging preparation that allows them to look at slices in mice as well as from human fetal tissue. The imaging is very impressive, and the findings are novel. I have two overall comments:

1. Though the data support a model where extra centrosomal nucleation occurs in the basal fibers,

there is no cause and effect. Could the authors delete a component of extra centrosomal nucleation and show that the EB3 comets are not formed under those conditions?

2. No mechanism is provided for the microtubules in the basal fibers that are pointing towards the ventricle. The authors should make an attempt to see what is there at the tip of the basal fiber that is giving rise to the phenotype?

Specific comments:

- The point of figure 1E is not clear
- The basal process is long, and the authors should do EB3 imaging in multiple segments (proximal, middle, distal) of this process to give a sense of the polarity in different parts of this process. Is the mixed polarity only seen in the very distal part of the basal process?
- Figure 4C: why are all the microtubules elongating only in one direction when there is a mixed polarity? I know that 80% of them are going that way according to the previous data, but one would think that this dual imaging would capture at least a few going the other way. This also relates to the previous point, suggesting that perhaps the mixed polarity is only seen in the very distal part of the basal fiber.
- I had a hard time following some parts of the text and figures because the authors use the words "apical" and "basal" to describe both the processes and the surfaces towards which the microtubules are elongating. This is confusing, and I suggest that the authors use different words to describe the directions in which the microtubules are elongating (for instance "pial" and "ventricular" surfaces).

Other comments:
- Typo on line 84 ("imagining")
- See 7,09 on line 129 (European comma for decimal?)
- Figure 5: I suggest bringing the quantification next to the images, so the authors don't have to say "see figure 5B, 5E" (sequence out of order).
- Is "cultivated" the right word for cultured neurons?
- The increased periodicity of microtubule-organizing centers in the human brain is interesting, but the authors explanation that this may be due to the greater lengths of the basal fibers in humans does not make sense to me. If the phenomenon is local (which is probably the case), as long as there are more numbers of microtubule-organizing centers in longer processes, that would probably work fine. Why would you need a greater density?

Reviewer #2 (Comments to the Authors (Required)):

In this report, Coquand and colleagues investigated the organization and growth of microtubules in mouse and human radial glial cells (RGs). By using in utero electroporation and vibratome brain slices, the authors described the orientation of microtubule growth. They found that while mouse apical processes display a basal-directed microtubule growth, basal processes have a mixed orientation in which there is about 15% which show an apical bias. Furthermore, they identified the swellings (varicosities) in basal processes as hotspots of acentrosomal (ɣ-TURC negative)

microtubule growth. Using human fetal tissue, they showed that basal microtubule polarized growth is conserved in human basal radial glia (bRGs). Finally, the authors showed that most microtubules in RG basal swellings grow from spots where the minus-end nucleator protein CAMSAP3 and trans-Golgi networks are localized.
Although this study is primarily descriptive, it addresses a relevant topic at the crossroads of the cortical development and cytoskeleton fields. The observations obtained by the authors may open new directions of research focused on the function and regulation of microtubule dynamics in RGs, which has currently been unexplored. However, I have concerns regarding the methodology and analyses of some results.

Major comments:

1. In panels of almost every figure (mice and human data)- the number of comets and/or swellings quantified are included as individual data points. However, for each analysis, the authors need to provide critical information regarding the extent of biological variability including: the number of RGs quantified, the number of brain slices, and the number of independent electroporations. This can be included in the figure legends. For rigor of this study it is important that all data is derived across multiple brain slices/experiments.

2. For the kymographs in Figure 1C, it appears that some short apically directed movements were not traced in the adjacent image. For example, some missing traces are evident at about 11 AM or 6 PM on a clock and comparable in size to some basally-directed movements which were traced. It is unclear how the authors defined which tracks to trace. If tracing of these "missing movements" is included in the quantifications in F-H, would it affect the proportion of apical vs. basal microtubule directed growth?

3. In Figure 3 the authors analyze bRGs of human fetal tissue. This reviewer appreciates the challenges of such experiments in human fetal tissue. Nonetheless, there are some missing analyses that the authors should include, to support their conclusions and make appropriate comparisons. First, in Figure 3F they need to report the rate of comet formation in the shafts (only the swellings is included here). This would be similar to what is reported in Figure 2D. Second, they need to show Sox2 staining to confirm the examined cells are indeed bRGs (the data are mentioned on line 144 but not shown). This is really important data, and ideally would be accompanied by other evidence. Third, and perhaps most important, it is unclear why the authors only report data on human bRGs and not on human aRGs. As a result, the comparisons they draw between mice and human are between two different cell types (aRG in mice and bRG in human). Thus, it is unclear if the shorter distances between swellings reported between mice and human in Figure 3I would hold true if they compared the same cell type. I would think these data may be analyzed from their existing movies, as electroporation would also target aRG.

4. In Figures 4 and 5, the authors demonstrate that swellings contain CAMSAP3 and TGN foci from which microtubules grow. While they also indicate these foci are present in the basal processes, this point is not well delineated which makes it confusing the nature of MTOCs throughout RGs. This is relevant since their data in Figure 1 demonstrate it isn't just swellings that have acentrosomal MTOCs, as also noted on line 244. Indeed, they note CAMSAP3 is located along the basal process and suggest comets grow from these loci (see lines 183 and 192). To clarify the extent to which CAMSAP3 foci are associated with MT growth in the basal process and how this compares to that in the swellings, the authors should quantify these data (CAMSAP3 and also EB3 comets from the CAMSAP3 loci in the basal process shaft). Likewise, to quantify the data shown in Figure 5C, they should measure GalNacT2+ foci in the basal process and also clarify its correlation with microtubule

growth. These quantifications would better define the role of these molecules as MTOCs and clarify the nature of microtubule growth in the basal process.

5. The conclusions in Figures 4 and 5 rely exclusively upon overexpression (either GFP-CAMSAP3 or GalNAcT2). The authors should validate these findings with endogenous labeling of both markers in RGs to confirm that overexpression is not an artifact.

Minor comments:

1. It is unclear why the authors use the term swellings. They may instead consider the term varicosities which I think is more common in the field.
2. Varicosities are especially prominent in mitotic RGCs as the basal process thins. Can the authors comment on whether the microtubule growth dynamics coincide with any specific cell cycle stages? Did they have resolution to visualize differences?
3. Line 39 (introduction): "neuroepithelial cells" could be a confusing terminology to describe RG cells because of neuroepithelial progenitor cells, that give rise to RG. I suggest to use instead the word "derived from"
4. Line 411 (Figure 3 legend): misspelling "fetal".
5. Figure 3I: If the quantification is distance between swelling, the number of samples (n) should not be swellings (see figure legend, line 426). Instead, the number of samples should reflect number of cells quantified, which is not detailed (please see also major point 1).
6. Figure 5B: quantification or information on how many swellings/cells were assessed for the cis-medial markers ManII and GMAP210, would further support the statement that were "undetectable outside the apical process" (please see also major point 1).
7. Do the phenomena regarding roles for CAMSAP3 and GalNacT2 happen across both basal and apically directed movements in RG cell swellings?
8. It would be helpful for the reader to provide a figure legend or an in-situ description for all the videos which are attached.
9. The materials and methods describes that Z-stacks were acquired for live-imaging experiments but it is not clear if the kymographs were generated from a maximum Z-stack projection or other settings.
10. Figure 3F only includes N=12 swellings. While it is unclear how many independent RGs and brain slices this includes, I have some concern about these data given how few swellings were quantified. Can the authors increase the numbers here?
11. In the abstract and throughout the paper the authors suggest that mixed polarity of basla processes is reminiscent of dendrites. However, this could be a bit exaggerated as there is far more bias in the basal process compared to dendrites. Please consider adjusting this, at least in the abstract.
12. It is unclear why the authors use the term Fmr1p as it should be FMRP?
13. The authors should use consistent nomenclature for %. (Eg,Line 130 is 89,6%; and line 132 is 65.7%).
14. For Figure 2E the authors may consider displaying these data as a stacked bar chart and perform chi-square analysis to compare classes. In addition, please clarify what the error bars represent and why they are different between each class.

Reviewer #3 (Comments to the Authors (Required)):

The polarity of the microtubule cytoskeleton is critical to many different aspects of cell structure and function. In the developing brain, neural stem cells called radial glial cells span the developing cortex. A long, thin basal process extends from the cell body, located near the ventricular zone, up to the pial surface. In this study, the authors characterise microtubule polarity in the basal process of radial glial cells (in mouse and human cortices. Interestingly, microtubule polarity in these basal processes is mixed, which is in contrast to the uniform microtubule polarity of the apical process. This difference in radial glial cell polarity, which is conserved between mice and humans, mirrors the differences in microtubule polarity in the dendrites and axons of mature neurons. The authors then go on to show that microtubules in the basal process frequently polymerise from neuritic swellings in which the microtubule minus-end stabiliser CAMSAP3 accumulates. The correlation of microtubule growth initiation sites and CAMPSAP3 in the swellings lead the authors to propose that these basal process swellings are enriched in non-centrosomal microtubule organizing centers (MTOCs).

The authors' conclusions are supported by their data, which are clearly and nicely presented in the figures. The inclusion of human cortical tissue is also quite nice as it shows that microtubule polarity in the basal processes is conserved. One major concern, however, is that the results are predominantly descriptive. While the authors' data are consistent with the idea that the basal process swellings house MTOCs, there is no functional support of this model. For example, it is ultimately not clear whether the swellings themselves are functionally relevant or critical to the organization of microtubules in the basal process; e.g. if the swellings were slimmed down or eliminated would this affect microtubule polarity? (One could imagine that the swellings may be necessary to trap MTOCs or that the swellings may form as the result of the MTOCs). On a related note, are the swellings stable or dynamic, and how many swellings are typically observed per basal process? It is also not clear what the molecular basis of the non-centrosomal MTOC would be. For example, which CAMSAP(s) are expressed in radial glial cells, and would knocking-down any of these proteins affect microtubule growth and/or polarity? Lastly, the actual proximity of the EB3 comet start sites to the GalNacT2-positive foci is difficult to ascertain from the images show in Figure 5. That a (potential) connection exists between the trans Golgi and microtubule growth is not a huge part of the manuscript, and, in general, the authors are careful about interpreting these data (like the CAMSAP3 results, there is no functional test of the relationship between the trans-Golgi compartment and EB3 comet start sites). Thus, they should consider removing the word "strong" to describe the association between the EB3 comets and GalNacT2-positive foci (line 220).

Minor concerns

1) The authors should take care with the references. There are several places where the reference does not support the statement and/or some references are missing (see below). Additionally, the authors should cite studies from the Vallee lab that have previously examined EB3-GFP in radial glial cells (e.g. Tsai et al. 2010; Tsai et al. 2007).
- Lines 6, 237: It would be more appropriate to cite a review in place of or in addition to Yau et al. 2016. Primary papers published earlier than Yau et al. 2016 are more appropriate to support the statements about microtubule polarity in neurons.
- Line 69: Ori-McKenney et al. 2012 does not look at dendritic microtubule polarity.
- Line 201: Additional references should be included, such as Horton and Ehlers 2003 (PMID: 12867502), Horton et al. 2005 (16337914), Ye et al. 2007 (PMID: 17719548).

2) In Figure 1, D and E, EB3 comets in different areas of the basal process are shown. Are there any

differences in microtubule growth or orientation based on position in the basal process? Which area(s) are quantified in Figure 1, F and H?

3) Figure 3G: Are the comets included for analysis just from swellings or also shafts? On a related note, in human bRGs, do more comets originate from swellings than shafts as in mouse (as shown in Figure 2D)?

4) Figure 4, A and B: Presumably N=nucleus; please add to legend.

5) Figures 4 and 5: What is the frequency (density) of CAMSAP3 and GalNacT2-positive foci in the basal process shaft? Do EB3 comets originate from these foci in shafts as well?

6) What is the polarity of the microtubules that emanate from the CAMSAP3 or GalNacT2-positive foci?

7) Minor comment: in Figure 4, the colors of the fluorescent proteins are reversed in the images (e.g. Emerald/GFP is shown in magenta, and mCherry is shown in green), which is slightly distracting. Was this done to best represent the signals, or to be consistent with color labelling in other figures?

JCB manuscript #202003151

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You will see that although all three reviewers have voiced enthusiasm for the study, they each raise a number of overlapping concerns which will need to be addressed before the paper would be suitable for publication in JCB.
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We wish to thank the reviewers for their comments and very constructive suggestions. Below is a point-by-point response to each comment.

Reviewer #1 (Comments to the Authors (Required)):

In this study the authors characterize the microtubule cytoskeleton in polarized radial glia. Looking at basal and apical fibers of these cells by EB3 imaging, they found that there was a difference in the polarity of microtubules in the apical and basal fibers, which was previously unknown. Specifically, microtubules in the apical fibers are uniformly polarized with minus ends directed towards the ventricular side, but they are of mixed polarity in the basal fibers. Furthermore, they found that focal varicosities in the basal fibers contained components that may lead to extra centrosomal nucleation of microtubules. All of this is shown in situ using an imaging preparation that allows them to look at slices in mice as well as from human fetal tissue. The imaging is very impressive, and the findings are novel. I have two overall comments:
1. Though the data support a model where extra centrosomal nucleation occurs in the basal fibers, there is no cause and effect. Could the authors delete a component of extra centrosomal nucleation and show that the EB3 comets are not formed under those conditions?

Single cell RNA-seq data indicate that, in radial glial progenitors, CAMSAP1, 2 & 3 are expressed, but CAMSAP 1 & 2 appear expressed at higher levels (Telley et al, 2019, Science). We therefore performed shRNA-mediated knockdown of CAMSAP 1 & 2 in radial glial progenitors. Efficient knockdown was validated at the mRNA level in Neuro2A cells using QPCR (**Supplemental figure 2E**). E14,5 embryonic brains were co-electroporated with CAMSAP1 shRNA construct, CAMSAP2 shRNA construct and EB3-GFP plasmid. Strikingly, CAMSAP knockdown led to a dramatic and fast destabilization of the entire basal process (**Supplemental figure 2D**). While this precluded an analysis of EB3 comets dynamics, this result highlights the importance of CAMSAP-mediated microtubule organization for the stability of radial glial cell basal process.

2. No mechanism is provided for the microtubules in the basal fibers that are pointing towards the ventricle. The authors should make an attempt to see what is there at the tip of the basal fiber that is giving rise to the phenotype?

The mechanism for microtubule polarity establishment is indeed an outstanding question and has been the subject of extensive work, in particular in neuronal cells. The first question to address is what makes most microtubules grow in the basal direction. As discussed in the manuscript, the establishment of a robust plus end out network in axons appears to depend on the Augmin complex. The following question is indeed to understand what makes a certain percentage of microtubules grow in the opposite direction. In dendrites, minus-end out microtubule organization was shown to depend on CAMSAP/Patronin. Whatever the exact mechanism in the basal process, we point out that it is not specific to the tip of the basal fiber. Indeed, as suggested by this reviewer (*see specific comment 2 for details*) we now show that bipolar microtubule organization is observed throughout most of the basal process (except at the apical-most part of the basal process, likely due to the proximity if the centrosome) (**Figure 1H, 1G**).

Specific comments:

- The point of figure 1E is not clear

We agree that in the submitted version, this montage could appear redundant with the kymograph of figure 1D. We however kept this figure to illustrate our new quantification of microtubule network polarity along the apicobasal axis (**Figure 1G** and *see following comment*).

- The basal process is long, and the authors should do EB3 imaging in multiple segments (proximal, middle, distal) of this process to give a sense of the polarity in different parts of this process. Is the mixed polarity only seen in the very distal part of the basal process?

This is indeed a very important information. We have now imaged growing microtubule plus ends in the apical-most, medial, and basal-most part of the basal process. Our results indicate that around 15% of apically-growing microtubules are observed throughout most of the basal process. However, we observe that in the apical-most part of the basal process, microtubule polarity is much more strongly biased towards basal growth (2,25% of apically-growing microtubules) (**Figure 1H, 1G**). We hypothesize that this is due to the relative proximity to the centrosome, which may still strongly influence polarity in this region. This result has important implications for the mechanisms of cargo entry into the basal process.

- Figure 4C: why are all the microtubules elongating only in one direction when there is a mixed polarity? I know that 80% of them are going that way according to the previous data, but one would think that this dual imaging would capture at least a few going the other way. This also relates to the previous point, suggesting that perhaps the mixed polarity is only seen in the very distal part of the basal fiber.

For these movies, we had to use an mcherry-tagged EB3 construct, which turns out to be much dimmer than the GFP construct in the brain. While most of these movies remained easily quantifiable, they often turned out too dim for kymograph representation. We chose this movie as it is representative regarding CAMSAP localization, and of good visual quality. It furthermore illustrates, in a single image, that both large CAMSAP puncta located within swellings and small CAMSAP puncta located outside are associated to new EB3 comets, as described in the text. Notably, this kymograph does reveal apically growing microtubules, even though not as abundantly as in the average condition. We have highlighted them on the figure.

- I had a hard time following some parts of the text and figures because the authors use the words "apical" and "basal" to describe both the processes and the surfaces towards which the microtubules are elongating. This is confusing, and I suggest that the authors use

different words to describe the directions in which the microtubules are elongating (for instance "pial" and "ventricular" surfaces).

We perfectly understand this concern, and have in fact tried different nomenclatures before the submission. However, our feeling is that the current nomenclature remains the most comprehensive way to refer RG cell polarity. We fear that terms such as “pial surface-directed growth” may confuse readers outside the field of neurodevelopment. We however revisited the text to identify instances where confusion could arise, in order to simplify and clearly state whether we are talking about basally-*directed microtubule growth* or about the basal *process.* Furthermore, we have indicated the apical and basal surface on each scheme that is opposed to the live imaging data.

Other comments:
- Typo on line 84 ("imagining")
We have corrected this.

- See 7,09 on line 129 (European comma for decimal?)
Indeed, we have used commas for decimal, and have changed them to points throughout the manuscript.

- Figure 5: I suggest bringing the quantification next to the images, so the authors don't have to say "see figure 5B, 5E" (sequence out of order).
We have tried this reorganization but it led to an unequilibrated figure, with space loss. We have therefore decide to leave the quantifications at the bottom of the figure.

- Is "cultivated" the right word for cultured neurons?
Cultured is indeed probably better. We have changed this.

- The increased periodicity of microtubule-organizing centers in the human brain is interesting, but the authors explanation that this may be due to the greater lengths of the basal fibers in humans does not make sense to me. If the phenomenon is local (which is probably the case), as long as there are more numbers of microtubule-organizing centers in longer processes, that would probably work fine. Why would you need a greater density?
Our reasoning was that microtubule-organizing centers might be more critical at greater distances from the main microtubule organizing center (the apically-localized centrosome). However, we agree that this hypothesis isn’t entirely solid (for several reasons such as the lack of density variation along the apico-basal axis in human samples) and was therefore deleted.

Reviewer #2 (Comments to the Authors (Required)):

In this report, Coquand and colleagues investigated the organization and growth of microtubules in mouse and human radial glial cells (RGs). By using in utero electroporation and vibratome brain slices, the authors described the orientation of microtubule growth. They found that while mouse apical processes display a basal-directed microtubule growth, basal processes have a mixed orientation in which there is about 15% which show an apical bias. Furthermore, they identified the swellings (varicosities) in basal processes as hotspots of acentrosomal (ɣ-TURC negative) microtubule growth. Using human fetal tissue, they showed that basal microtubule polarized growth is conserved in human basal radial glia (bRGs). Finally, the authors showed that most microtubules in RG basal swellings grow from spots where the minus-end nucleator protein CAMSAP3 and trans-Golgi networks are localized. Although this study is primarily descriptive, it addresses a relevant topic at the crossroads of the cortical development and cytoskeleton fields. The observations obtained by the authors may open new directions of research focused on the function and regulation of microtubule dynamics in RGs, which has currently been unexplored. However, I have concerns regarding

the methodology and analyses of some results.

Major comments:

1. In panels of almost every figure (mice and human data)- the number of comets and/or swellings quantified are included as individual data points. However, for each analysis, the authors need to provide critical information regarding the extent of biological variability including: the number of RGs quantified, the number of brain slices, and the number of independent electroporations. This can be included in the figure legends. For rigor of this study it is important that all data is derived across multiple brain slices/experiments.

We agree this is indeed a very important point. We have now added, for each experiment, the number of comets, swellings, cells, brain slices and/or independent samples.

2. For the kymographs in Figure 1C, it appears that some short apically directed movements were not traced in the adjacent image. For example, some missing traces are evident at about 11 AM or 6 PM on a clock and comparable in size to some basally-directed movements which were traced. It is unclear how the authors defined which tracks to trace. If tracing of these "missing movements" is included in the quantifications in F-H, would it affect the proportion of apical vs. basal microtubule directed growth?

This is an important point that needed clarification. The outline of the traces was done to illustrate the figure, but it indeed appears that some short tracks are missing. We have now corrected this to include all observed tracks. For quantifications, the identification of comets was directly done on the movies, which turns out to be more sensitive and to allow easier spotting of the short events. Kymographs were used as a secondary and confirmatory tool. We have now better described this in the methods section.

3. In Figure 3 the authors analyze bRGs of human fetal tissue. This reviewer appreciates the challenges of such experiments in human fetal tissue. Nonetheless, there are some missing analyses that the authors should include, to support their conclusions and make appropriate comparisons. First, in Figure 3F they need to report the rate of comet formation in the shafts (only the swellings is included here). This would be similar to what is reported in Figure 2D.

We have now quantified the rate of comet formation in both swellings and shafts of human bRG cells. This analysis reveals that the rate of comet formation is extremely similar between mouse and human shafts, as well as between mouse and human swellings (**Figure 3F**). Accordingly, the ratio between the two structures is conserved between mouse and human (around 10-fold difference).

Second, they need to show Sox2 staining to confirm the examined cells are indeed bRGs (the data are mentioned on line 144 but not shown). This is really important data, and ideally would be accompanied by other evidence.

We now provide a Sox2 staining of electroporated human bRG cells (**Supplemental figure 1A**). They are further identified as bRG cells and not aRG cells due to their position in the tissue and lack of contact with the ventricular surface. Moreover, we now present long-term live imaging data showing that these cells perform mitotic somal translocation (MST), a typical bRG behavior consisting in fast movement of the soma followed by cell division (**Supplemental figure 1B**).

Third, and perhaps most important, it is unclear why the authors only report data on human bRGs and not on human aRGs. As a result, the comparisons they draw between mice and human are between two different cell types (aRG in mice and bRG in human). Thus, it is unclear if the shorter distances between swellings reported between mice and human in Figure 3I would hold true if they compared the same cell type. I would think these data may be analyzed from their existing movies, as electroporation would also target aRG.

Electroporation indeed primarily targets aRG cells. We however note a very low amount of electroporated aRG cells after 48 hours (the time for robust GFP expression in these cells),

with the vast majority of cells having a bRG, intermediate progenitor, or neuronal identity. This is a consequence of apical surface disorganization after 2 days of culture on filter, and is something we also observe for mouse brain slices. This empirical observation facilitated the analysis of bRG cells, but also impaired us from analyzing human aRG cells.

4. In Figures 4 and 5, the authors demonstrate that swellings contain CAMSAP3 and TGN foci from which microtubules grow. While they also indicate these foci are present in the basal processes, this point is not well delineated which makes it confusing the nature of MTOCs throughout RGs. This is relevant since their data in Figure 1 demonstrate it isn't just swellings that have acentrosomal MTOCs, as also noted on line 244. Indeed, they note CAMSAP3 is located along the basal process and suggest comets grow from these loci (see lines 183 and 192). To clarify the extent to which CAMSAP3 foci are associated with MT growth in the basal process and how this compares to that in the swellings, the authors should quantify these data (CAMSAP3 and also EB3 comets from the CAMSAP3 loci in the basal process shaft). Likewise, to quantify the data shown in Figure 5C, they should measure GalNacT2+ foci in the basal process and also clarify its correlation with microtubule growth. These quantifications would better define the role of these molecules as MTOCs and clarify the nature of microtubule growth in the basal process.

We indeed observe most EB3 comets emanating from the swellings, and also a fraction from the shaft. Likewise, we observe a concentration of CAMSAP and GalNacT2 in swellings, but also a fraction in the shaft. In response to the reviewer's comment, we now present the association of EB3 comets with these two markers in the entire basal process, in the swellings specifically, and in the rest of the shaft specifically. We show that, overall, 74,4% of EB3 comets emanate from CAMSAP3 foci. In the swellings, this goes up to 81,6%, while in the shaft only 61 % of EB3 comets emanate from CAMSAP3 foci (**Figure 4E**). Likewise, 72% of EB3 comets emanate from GalNacT2 foci throughout the basal process. In the swellings, this goes up to 81,3% while in the shaft it only reaches 42,9% (**Figure 5F**). This data highlights the strong association between, EB3 comet formation, swellings, and CAMSAP3 or GalnacT2 foci. As microtubules grow and shrink over much longer distances in the shafts, the CAMSAP3-independent EB3 comets that form in this region may represent rescue events.

5. The conclusions in Figures 4 and 5 rely exclusively upon overexpression (either GFP-CAMSAP3 or GalNAcT2). The authors should validate these findings with endogenous labeling of both markers in RGs to confirm that overexpression is not an artifact.

We now present immunostainings for CAMSAP1 and CAMSAP2 in mouse and human brain slices, and GalNAcT2 in human brain slices. All three factors are found within basal process swellings (**Supplemental Figures 2B and 3A**). We note that the tissue being extremely dense, signal is also observed around the cells. We nevertheless robustly detect these factors within swellings. We have decided to present the "raw" immunostaining rather than a segmented image, which we feel illustrates best the experiment. We have also stained *in vitro* cultured human radial glial cells. While these cells do not form swellings, we reproducibly detect CAMSAP and GalNacT2 in their processes. Together with the localization of the tagged protein, this data indicates that CAMSAPs and GalNacT2 localize to basal process swellings in mouse and human RG cells.

Minor comments:

1. It is unclear why the authors use the term swellings. They may instead consider the term varicosities which I think is more common in the field.
Indeed, varicosities may be more commonly used. We have replaced swellings by varicosities.

2. Varicosities are especially prominent in mitotic RGCs as the basal process thins. Can the authors comment on whether the microtubule growth dynamics coincide with any specific cell cycle stages? Did they have resolution to visualize differences?

Basal process thinning during mitosis can indeed make varicosities more prominent. Cell cycle regulation is a very interesting point for which we do not have an answer. In many cell types, mitotic spindle formation is accompanied by an important reduction of “cytoplasmic” microtubule nucleation. Whether this is the case in the highly elongated RG cells is unclear.

3. Line 39 (introduction): "neuroepithelial cells" could be a confusing terminology to describe RG cells because of neuroepithelial progenitor cells, that give rise to RG. I suggest to use instead the word "derived from"
Indeed. We changed the sentence to “Apical radial glial (aRG) cells, also known as vRGs, have an epithelial identity and are present in all mammalian species” as we wanted to emphasize that aRG cells are epithelial.

4. Line 411 (Figure 3 legend): misspelling "fetal".
We have changed all instances of foetal to use this spelling.

5. Figure 3I: If the quantification is distance between swelling, the number of samples (n) should not be swellings (see figure legend, line 426). Instead, the number of samples should reflect number of cells quantified, which is not detailed (please see also major point 1).
We now report the number of cells for this experiment.

6. Figure 5B: quantification or information on how many swellings/cells were assessed for the cis-medial markers ManII and GMAP210, would further support the statement that were "undetectable outside the apical process" (please see also major point 1).
For GMAP210, 235 swellings out 27 cells were analyzed (coming from 2 independent electroporations). For ManII, 381 swellings out of 36 were analyzed (coming from 3 independent electroporations). In each case, although the signal was very strong in the Golgi apparatus, no signal was observed in swellings. We have now added this information in the figure legend.

7. Do the phenomena regarding roles for CAMSAP3 and GalNacT2 happen across both basal and apically directed movements in RG cell swellings?
This point was also raised by reviewer 3 (minor point 6). We have now measured the polarity of EB3 comets emanating from CAMSAP3 or GalNacT2-positive foci. This analysis indicated that the polarity of CAMSAP3 or GalNacT2-associated EB3 comets is similar to overall polarity reported in figure 1 (**Supplemental Figures 2A and 3C**).

8. It would be helpful for the reader to provide a figure legend or an in-situ description for all the videos which are attached.
We apologize for omitting this. It has been added.

9. The materials and methods describes that Z-stacks were acquired for live-imaging experiments but it is not clear if the kymographs were generated from a maximum Z-stack projection or other settings.
All presented movies (and related kymographs) were generated following maximum projections of the Z-stacks (15-20 planes, 1μ step size). This has now been better detailed in the methods.

10. Figure 3F only includes N=12 swellings. While it is unclear how many independent RGs and brain slices this includes, I have some concern about these data given how few swellings were quantified. Can the authors increase the numbers here?
We have increased the number of counted human bRG varicosities. The data presented here corresponds to 2 independent human fetal brain samples, in wich 30 varicosities were live imaged and quantified (**Figure 3F**). 25 shafts were also live imaged and quantified.

11. In the abstract and throughout the paper the authors suggest that mixed polarity of basal

processes is reminiscent of dendrites. However, this could be a bit exaggerated as there is far more bias in the basal process compared to dendrites. Please consider adjusting this, at least in the abstract.
We find that 15% of microtubules are oriented apically, while in mammalian dendrites 30% of microtubules grow inwards. We feel these numbers do support a comparison between the two systems, which are both bipolar, have a majority of microtubules growling outwards, and a minority of microtubes growing inwards. We therefore used terms such as “reminiscent”, which we feel are not to strong. We have considered the term “bipolar microtubule” network in the title (instead of dendritic-like), but fear this may convey the idea of a 50-50 polarity. We do understand the reviewer’s comment, and have been careful throughout the manuscript to mention the similarity between the two systems, without making any over statement.

12. It is unclear why the authors use the term Fmr1p as it should be FMRP?
We have corrected this to FMRP

13. The authors should use consistent nomenclature for %. (Eg,Line 130 is 89,6%; and line 132 is 65.7%).
We have changed comas for points throughout the manuscript.

14. For Figure 2E the authors may consider displaying these data as a stacked bar chart and perform chi-square analysis to compare classes. In addition, please clarify what the error bars represent and why they are different between each class.
The error bars of figure 2E (now 2F) represent SD and are different as each percentage can vary to a different extent from one experiment to the other (% of tangential comets is almost constant, while apically and basally directed comets vary more). We therefore favor this representation.

Reviewer #3 (Comments to the Authors (Required)):

The polarity of the microtubule cytoskeleton is critical to many different aspects of cell structure and function. In the developing brain, neural stem cells called radial glial cells span the developing cortex. A long, thin basal process extends from the cell body, located near the ventricular zone, up to the pial surface. In this study, the authors characterise microtubule polarity in the basal process of radial glial cells (in mouse and human cortices. Interestingly, microtubule polarity in these basal processes is mixed, which is in contrast to the uniform microtubule polarity of the apical process. This difference in radial glial cell polarity, which is conserved between mice and humans, mirrors the differences in microtubule polarity in the dendrites and axons of mature neurons. The authors then go on to show that microtubules in the basal process frequently polymerise from neuritic swellings in which the microtubule minus-end stabiliser CAMSAP3 accumulates. The correlation of microtubule growth initiation sites and CAMPSAP3 in the swellings lead the authors to propose that these basal process swellings are enriched in non-centrosomal microtubule organizing centers (MTOCs).

The authors' conclusions are supported by their data, which are clearly and nicely presented in the figures. The inclusion of human cortical tissue is also quite nice as it shows that microtubule polarity in the basal processes is conserved.One major concern, however, is that the results are predominantly descriptive. While the authors' data are consistent with the idea that the basal process swellings house MTOCs, there is no functional support of this model. For example, it is ultimately not clear whether the swellings themselves are functionally relevant or critical to the organization of microtubules in the basal process; e.g. if the swellings were slimmed down or eliminated would this affect microtubule polarity? (One could imagine that the swellings may be necessary to trap MTOCs or that the swellings may form as the result of the MTOCs).

We agree that swellings may trap MTOCs, or may form as a consequence MTOC presence. Nevertheless, we fell this does not alter the conclusion of the study, which is that MTOCs are located within swellings. We currently lack the tools and knowledge to eliminate swellings. We suspect these structures are important sites of adhesion to the ECM, and further work may provide us with methods to alter swelling formation.

On a related note, are the swellings stable or dynamic, and how many swellings are typically observed per basal process?

We performed long term live imaging to evaluate swelling stability. These experiments reveal that swellings are stable, most of the time remaining present for the entire duration of the movie (24 hours) (**Figure 2C**). The number of swelling per basal process is variable as it is a function of the size of the basal process. In aRG cells, these vary substantially along the antero-posterior axis. The variability is even higher in bRG cells, which are scattered throughout the sub-ventricular zone (and therefore located at different distances to the pial surface). We therefore feel that providing the average density of swelling in both cell types (**Figure 3I**) is more informative.

It is also not clear what the molecular basis of the non-centrosomal MTOC would be. For example, which CAMSAP(s) are expressed in radial glial cells, and would knocking-down any of these proteins affect microtubule growth and/or polarity?

In the mouse developing cortex, CAMSAP 1, 2 and 3 all appear expressed but single cell RNAseq data indicates that CAMSAP 1 & 2 are expressed at the highest level (Telley et al, 2019, Science). We therefore performed double shRNA-mediated knock-down of CAMSAP 1 & 2 in aRG cells coexpressing EB3-GFP. KD efficiency was validated at the mRNA level in mouse Neuro2A cells (**Supplemental Figure 2E**). These experiments revealed that, as soon as 1 day post-electroporation, the basal processes of aRG cells were completely destabilized. We note that Nocodazol treatment (which we have performed while trying to do regrowth experiments) also leads to a rapid destabilization of the basal process (not shown). While these experiments did not allow us to directly monitor EB3 comet formation in varicosities, they are in favor of a critical role of CAMSAPs in basal process microtubule organization.

Lastly, the actual proximity of the EB3 comet start sites to the GalNacT2-positive foci is difficult to ascertain from the images show in Figure 5. That a (potential) connection exists between the trans Golgi and microtubule growth is not a huge part of the manuscript, and, in general, the authors are careful about interpreting these data (like the CAMSAP3 results, there is no functional test of the relationship between the trans-Golgi compartment and EB3 comet start sites). Thus, they should consider removing the word "strong" to describe the association between the EB3 comets and GalNacT2-positive foci (line 220).
We agree with the reviewer's comment and have changed the text accordingly.

Minor concerns

1) The authors should take care with the references. There are several places where the reference does not support the statement and/or some references are missing (see below). Additionally, the authors should cite studies from the Vallee lab that have previously examined EB3-GFP in radial glial cells (e.g. Tsai et al. 2010; Tsai et al. 2007).
We have now added these citations.

- Lines 6, 237: It would be more appropriate to cite a review in place of or in addition to Yau et al. 2016. Primary papers published earlier than Yau et al. 2016 are more appropriate to support the statements about microtubule polarity in neurons.
We now cite a review describing the work on microtubule polarity in neurons (Baas & Lin, 2011).

- Line 69: Ori-McKenney et al. 2012 does not look at dendritic microtubule polarity.
We have removed this citation from this paragraph. The paper is now cited later in the discussion.

- Line 201: Additional references should be included, such as Horton and Ehlers 2003 (PMID: 12867502), Horton et al. 2005 (16337914), Ye et al. 2007 (PMID: 17719548).
These are indeed important citations, which we have now added.

2) In Figure 1, D and E, EB3 comets in different areas of the basal process are shown. Are there any differences in microtubule growth or orientation based on position in the basal process? Which area(s) are quantified in Figure 1, F and H?
This question was also asked by reviewer 1 (specific comment 2). Previous quantifications were performed throughout the basal process, excluding the region immediately adjacent to the nucleus. We have now imaged growing microtubule plus ends in the apical-most, medial, and basal-most part of the cell. This analysis reveals that microtubule polarity is uniform throughout most of the basal process (**Figure 1F, 1G**). However, in the apical-most part of the basal process, microtubules are much more strongly unipolar, similar to what we observe in the apical process. We hypothesize that this is due to the relative proximity to the centrosome, which still strongly influences microtubule organization in this region.

3) Figure 3G: Are the comets included for analysis just from swellings or also shafts?
On a related note, in human bRGs, do more comets originate from swellings than shafts as in mouse (as shown in Figure 2D)?
The data in figure 3G represents overall polarity of all EB3 comets (shaft and swellings), as in figure 1F. This has been clarified in the legend. The second question was also asked by reviewer 2 (major comment 3). We have now quantified the rate of comet formation in the shaft of human bRG cells, which reveals that the difference in EB3 comet formation rates between shafts and varicosities is indeed very similar to what we observed in mouse (**Figure 3F**).

4) Figure 4, A and B: Presumably N=nucleus; please add to legend.
We have added this to the legend.

5) Figures 4 and 5: What is the frequency (density) of CAMSAP3 and GalNacT2-positive foci in the basal process shaft?
We have measured the frequency of CAMSAP3 and GalNacT2 foci in the basal process shaft. This analysis revealed that CAMSAP3 foci are observed every 6,4 µm, while GalNacT2 foci are observed every 8,8 µm. This has been added to the text.
Do EB3 comets originate from these foci in shafts as well?
We have now quantified EB3 comet association to CAMSAP3 and GalNacT2 in swellings and in shafts (see also Reviewer 2, major comment 4). We show that, overall, 74,4% of EB3 comets emanate from CAMSAP3 foci. In the swellings, this goes up to 81,6%, while in the shaft only 61 % of EB3 comets emanate from CAMSAP3 foci. Likewise, 72% of EB3 comets emanate from GalNacT2 foci throughout the basal process. In the swellings, this goes up to 81,3% while in the shaft it only reaches 42,9% (**Figures 4E & 5F**). This data highlights the strong association between EB3 comet formation, swellings, and CAMSAP3 or GalnacT2 foci.

6) What is the polarity of the microtubules that emanate from the CAMSAP3 or GalNacT2-positive foci?
We have now measured the polarity of EB3 comets specifically emanating from CAMSAP3 or GalNacT2-positive foci. This analysis indicates that this polarity is similar to the overall EB3 polarity (**Supplemental Figures 2A & 3C**).

7) Minor comment: in Figure 4, the colors of the fluorescent proteins are reversed in the

images (e.g. Emerald/GFP is shown in magenta, and mCherry is shown in green), which is slightly distracting. Was this done to best represent the signals, or to be consistent with color labelling in other figures?

This was done to be consistent with the other figures (outline of the cell is always shown in green throughout the manuscript).

January 25, 2021

Re: JCB manuscript #202003151R

Dr. Alexandre D Baffet
Institut Curie, PSL Research University, CNRS UMR144, Paris, France
12, rue Lhomond
Paris 75005
France

Dear Dr. Baffet,

Thank you for submitting your revised manuscript entitled "A dendritic-like microtubule network is organized from basal fiber varicosities in neural stem cells". We apologize for the delay in providing you with a decision.
In any case, the manuscript has been seen by the original reviewers whose full comments are appended below. Two of the reviewers continue to be overall positive about the work and all three reviewers believe that the manuscript has made substantial progress. However, they also agree that some important issues remain and, thus, we are unable to publish the paper in its current form.

You will see that the reviewers feel that the data linking CAMSAP1/2 with EB3 comet dynamics remains insufficient to support your conclusions. The reviewers also raise some concerns about the convincingness and thoroughness of the colocalization/staining data and the relative roles of the different CAMSAP proteins. Both we and the reviewers feel that these issues need to be conclusively addressed via new experiments. Thus, if you wish to publish this work quickly, it may be in your best interests to submit the manuscript elsewhere.

As you may know, our general policy is that papers are considered through only one revision cycle. However, given that the reviewers have voiced significant enthusiasm for the underlying premise of the study, we are willing to make a rare exception to this rule and allow you to submit one final revision, provided that you are able to address the reviewer concerns with new data. While we appreciate that concurrent knockdown of CAMSAP1 and 2 results in basal process destabilization, the reviewers have suggested other methods to at least reinforce some of the related conclusions. Specifically, we feel that it may be possible to examine the effects of knockdown of CAMSAP1 and 2 individually, as suggested by reviewer #2. If this proves unsuccessful (and/or reveals no phenotype due to compensation), we would also recommend that you examine CAMSAP1 and 2 colocalization with EB3 comets, as suggested by reviewer #3. As also suggested by reviewer #2, please provide the endogenous CAMSAP3 staining and the zoomed-out images of the CAMSAP1/2 and GALNACT2 staining. As you will see, reviewer #3 has recommended that you examine the effects of CAMSAP3 knockdown. While we agree with the reviewers that this would add further support for your conclusions and would encourage you to consider adding such data if it is practical, we will not require that experiment for resubmission.
Please note that I will expect to make a final decision without additional reviewer input upon resubmission.

Please submit the final revision as quickly as time allows (within two months, preferably, but if this proves impossible, please let us know) along with a rebuttal that includes a point by point response

to the remaining reviewer comments.

Also note that we are happy to transfer the reviewer comments to any other journal. Thus, if you would instead prefer to take the manuscript elsewhere and transfer the comments, just let us know.

Regardless of what you decide, thank you for this interesting contribution to Journal of Cell Biology. You can contact me or the scientific editor listed below at the journal office with any questions, cellbio@rockefeller.edu.

Sincerely,

Erika Holzbaur, PhD
Monitoring Editor
Journal of Cell Biology

Tim Spencer, PhD
Executive Editor
Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

The authors have made attempts to address my concerns, though the collapse of the axon upon CAMSAP1/2 knockout did not address a key issue that I had (also Rev #3).

In Fig. 1F (additional analyses done as per my review), the authors use apical/basal for defining different segments within the basal region. First, the same word should not be used to mean two different things (proximal/middle/distal to soma may make more sense, when talking about segments of the basal process). Second, the interpretation of 1G "..around 15% of apically-growing microtubules are observed throughout most of the basal process" is not appropriate. It seems clear that there are much fewer apically-directed microtubules in the basal process as we get closer to the soma. This cannot be explained by the model that all the microtubules in the basal process are originating acentrosomally from the varicosities. The data suggest that there be nucleation in the soma, or perhaps there are some microtubules that extending from the centrosome at the tip of the apical process after all (the authors exclude this as a mechanism, but given these data it cannot be excluded). Is the frequency of varicosities periodic throughout the basal process? In the schematic the authors have drawn periodic varicosities are shown along the entire length, but I don't see a picture of the entire axon from the soma to the basal surface (GFP-filled), where one can see this.

There is still one "cultivated" left in the text.

Reviewer #2 (Comments to the Authors (Required)):

In the revised version of the manuscript, the authors added valuable information that improves the quality of their work and further support their findings. This is a nice and thorough paper. Although descriptive it addresses an important question in the field and opens up new research directions. I

have a few remaining concerns, raised by these new data.
1. In response to the request to validate overexpression with endogenous staining, the authors include new stainings for CAMSAP1 and 2 and GALNACT2. However, the overexpression experiments in Figure 4 rely upon CAMSAP3. In response to reviewer 1, the authors imply that CAMSAP3 is expressed at lower levels than 1 and 2. Therefore, they really need CAMSAP3 endogenous staining to verify the enriched subcellular localization in varicosities to support that this is biologically relevant.
2. The stainings for CAMSAP1, 2, and GALNACT2 are not very convincing in tissue. The authors should include zoomed out views to appreciate the cortical staining, and ideally provide validation in knockdown brains (which they have in Figure S2).
3. The reviewers asked for functional evidence of the CAMSAP proteins in radial glia. The authors include data that dual knockdown severely impairs radial glial basal fiber formation and thus prohibits analysis of microtubules. However, with this in mind, why not perform analyses of single knockdown of either CAMSAP1 or 2 alone, which may overcome this robust phenotype? This seems straightforward.
4. Minor: In the revised version of the manuscript, the authors have included the number of cells quantified in all the experiments. However, most of them are still lacking information on how many independent experiments were performed and quantified (i.e. figure 2, figure 3 for mouse samples, figure 4, figure 5). Only figure 1 specifies that cells come from at least 3 mice and in figure 3 that the human data comes from 2 independent human fetal brains.
5. Minor: The authors need to add in new experiments to the methods, including Sox2 staining conditions, and culturing of radial glia with basal fibers.

Reviewer #3 (Comments to the Authors (Required)):

The authors nicely responded to the reviewers' comments and have conducted additional analysis and experiments, most importantly to address a major concern about the potential role of CAMSAP proteins (or other components of the basal process varicosities) in regulating microtubule growth. It is great that the authors conducted CAMSAP1 and CAMSAP2 loss-of-function experiments; unfortunately, however, these new experiments do not provide insight into the potential mechanism governing microtubule growth and polarity in the basal process. Thus, the manuscript remains largely descriptive with a model based on correlative experiments.

Overall, there is a bit of a disconnect between the descriptive and functional analysis of the relationship between CAMSAP proteins and EB3 comets (descriptive correlation being done with CAMSAP3 and functional analysis with CAMSAP1 and CAMSAP2). Findings from different groups suggest that the CAMSAP proteins, while related, are likely to play distinct roles in developing neurons, so it would be best to not treat these proteins as interchangeable. The authors state that CAMSAP1 and CAMSAP2 are the predominant CAMSAP proteins expressed in the RG cells (lines 169-170), but they analyze the co-localization of CAMSAP3 with EB3 comets. If the authors feel that CAMSAP1 and CAMSAP2 are the main players, then it would make sense to analyze the relative localization of these two CAMSAP proteins with EB3 comets. Conversely, the authors did not analyze whether the loss of CAMSAP3 has any effect on EB3 comets or microtubules growth and polarity. Even though CAMSAP3 expression may be lower, it might still play an important role in microtubule dynamics and organization (e.g. Pongrakhananon et al. 2018, PMID 30190432).

Minor comments:

1. The significance of the varicosities is also still unclear. There is a correlation between EB3 comet initiation and CAMSAP3 (and GalNacT2) in the shaft, but this correlation is much stronger in the varicosities, which suggests that CAMSAP3 (or CAMSAP1/2) alone is not sufficient.

2. The over-expression of CAMSAP3 does not necessarily equate to CAMSAP3 gain-of-function, but it would be helpful for the authors to comment on whether EB3 comet behavior is similar between neurons expressing and not expressing CAMSAP3-GFP. Also, it would be helpful to see the expression/distribution of endogenous CAMPSAP3.

3. Regarding naming different parts of the basal process (e.g. Figure 1F): Another reviewer commented on the different usages of basal, apical, etc. One thought: Would labeling the three sections of the basal process as "distal," "medial," and "proximal" be preferable to basal, medial, apical? This isn't a major concern.

4. In Supplemental Figure 2B: Please specify that CAMSAP1/2 is shown in B&W channel.

2nd Revision - Authors' Response to Reviewers: April 2, 2021

JCB manuscript #202003151R

Dear Dr. Baffet,

Thank you for submitting your revised manuscript entitled "A dendritic-like microtubule network is organized from basal fiber varicosities in neural stem cells". We apologize for the delay in providing you with a decision.
In any case, the manuscript has been seen by the original reviewers whose full comments are appended below. Two of the reviewers continue to be overall positive about the work and all three reviewers believe that the manuscript has made substantial progress. However, they also agree that some important issues remain and, thus, we are unable to publish the paper in its current form.

You will see that the reviewers feel that the data linking CAMSAP1/2 with EB3 comet dynamics remains insufficient to support your conclusions. The reviewers also raise some concerns about the convincingness and thoroughness of the colocalization/staining data and the relative roles of the different CAMSAP proteins. Both we and the reviewers feel that these issues need to be conclusively addressed via new experiments. Thus, if you wish to publish this work quickly, it may be in your best interests to submit the manuscript elsewhere.

As you may know, our general policy is that papers are considered through only one revision cycle. However, given that the reviewers have voiced significant enthusiasm for the underlying premise of the study, we are willing to make a rare exception to this rule and allow you to submit one final revision, provided that you are able to address the reviewer concerns with new data. While we appreciate that concurrent knockdown of CAMSAP1 and 2 results in basal process destabilization, the reviewers have suggested other methods to at least reinforce some of the related conclusions. Specifically, we feel that it may be possible to examine the effects of knockdown of CAMSAP1 and 2 individually, as suggested by reviewer #2. If this proves unsuccessful (and/or reveals no phenotype due to compensation), we would also recommend that you examine CAMSAP1 and 2 colocalization with EB3 comets, as suggested by reviewer #3. As also suggested by reviewer #2, please provide the endogenous CAMSAP3 staining and the zoomed-out images of the CAMSAP1/2 and GALNACT2 staining. As you will see, reviewer #3 has recommended that you examine the effects of CAMSAP3 knockdown. While we agree with the reviewers that this would add further support for your conclusions and would encourage you to consider adding such data if it is practical, we will not require that experiment for resubmission.
Please note that I will expect to make a final decision without additional reviewer input upon resubmission.

Please submit the final revision as quickly as time allows (within two months, preferably, but if this proves impossible, please let us know) along with a rebuttal that includes a point by point response to the remaining reviewer comments.

We thank the reviewers for their comments and very constructive suggestions. Below is a point-by-point response to each comment.

Reviewer #1 (Comments to the Authors (Required)):

The authors have made attempts to address my concerns, though the collapse of the axon upon CAMSAP1/2 knockout did not address a key issue that I had (also Rev #3).
We have now performed CAMSAP1 & 2 knockout individually (please see reviewer 2 comment).

In Fig. 1F (additional analyses done as per my review), the authors use apical/basal for defining different segments within the basal region. First, the same word should not be used to mean two different things (proximal/middle/distal to soma may make more sense, when talking about segments of the basal process).

We agree that this nomenclature is more understandable, and have revised it.

Second, the interpretation of 1G "..around 15% of apically-growing microtubules are observed throughout most of the basal process" is not appropriate. It seems clear that there are much fewer apically-directed microtubules in the basal process as we get closer to the soma. This cannot be explained by the model that all the microtubules in the basal process are originating acentrosomally from the varicosities. The data suggest that there be nucleation in the soma, or perhaps there are some microtubules that extending from the centrosome at the tip of the apical process after all (the authors exclude this as a mechanism, but given these data it cannot be excluded).
We entirely agree with this and apologize if this was confusing. We do believe that the unipolar microtubule organization seen proximally is likely due to the proximity to the centrosome. We have clarified this in the text "*Bipolar microtubule organization was observed in the distal and medial parts of the basal process, but not in the proximal part where the network was largely unipolar, likely due to the proximity of the centrosome*"

Is the frequency of varicosities periodic throughout the basal process? In the schematic the authors have drawn periodic varicosities are shown along the entire length, but I don't see a picture of the entire axon from the soma to the basal surface (GFP-filled), where one can see this.
We have not detected any periodicity for the varicosities. As seen in figure 3I, there is a great variability of varicosity-to-varicosity distance (even in human, although the scale used makes it less visible, as the average distance is much smaller). A low-resolution image of entire basal processes can be seen in figure 2C. We have indicated in the text that the distance between varicosities was very variable.

There is still one "cultivated" left in the text.
We have replaced it.

Reviewer #2 (Comments to the Authors (Required)):

In the revised version of the manuscript, the authors added valuable information that improves the quality of their work and further support their findings. This is a nice and thorough paper. Although descriptive it addresses an important question in the field and opens up new research directions. I have a few remaining concerns, raised by these new data.

1. In response to the request to validate overexpression with endogenous staining, the authors include new stainings for CAMSAP1 and 2 and GALNACT2. However, the overexpression experiments in Figure 4 rely upon CAMSAP3. In response to reviewer 1, the authors imply that CAMSAP3 is expressed at lower levels than 1 and 2. Therefore, they really need CAMSAP3 endogenous staining to verify the enriched subcellular localization in varicosities to support that this is biologically relevant.
We now provide CAMSAP3 endogenous stainings (Fig. S2A and C). Please also see the following comment.

2. The stainings for CAMSAP1, 2, and GALNACT2 are not very convincing in tissue. The authors should include zoomed out views to appreciate the cortical staining, and ideally provide validation in knockdown brains (which they have in Figure S2).
We now provide zoomed out versions of the stainings. It was not possible to quantitatively demonstrate signal reduction in KD cells. As is often the case in this very dense tissue, the stainings are very busy (the density of basal fibers is high, and these factors are also expressed in the surrounding neurons). Signal intensity argues against backgrounds staining, and we believe that the very specific localization of the GFP-tagged construct, together with the transcriptomic data, argues for specific basal process localization of CAMSAP and TGN proteins.

3. The reviewers asked for functional evidence of the CAMSAP proteins in radial glia. The authors include data that dual knockdown severely impairs radial glial basal fiber formation and thus prohibits

analysis of microtubules. However, with this in mind, why not perform analyses of single knockdown of either CAMSAP1 or 2 alone, which may overcome this robust phenotype? This seems straightforward.
We have now performed live imaging of EB3-GFP in CAMSAP1 and CAMSAP2 single knockdown cells. Unlike in the double KD, basal processes were still present, although their morphology was slightly affected, especially following CAMSAP1 KD (Fig. 5A). These data indicate partial compensation between CAMSAP1 and CAMSAP2 for basal process architecture.

We observed a substantial reduction in EB3 comet formation within varicosities of both CAMSAP1 and CAMSAP2 shRNA-transfected cells (Fig. 5 B & C). EB3 comet formation inside shafts was not significantly affected (Fig. 5 B & D). These results indicate that CAMSAP1 & 2 are required for EB3 comet formation within varicosities of the basal process in RG cells.

4. Minor: In the revised version of the manuscript, the authors have included the number of cells quantified in all the experiments. However, most of them are still lacking information on how many independent experiments were performed and quantified (i.e. figure 2, figure 3 for mouse samples, figure 4, figure 5). Only figure 1 specifies that cells come from at least 3 mice and in figure 3 that the human data comes from 2 independent human fetal brains.
We have now added the number of independent experiments for each figure.

5. Minor: The authors need to add in new experiments to the methods, including Sox2 staining conditions, and culturing of radial glia with basal fibers.
We have added the reference and dilution of the SOX2 antibody (immunostaining in human brain slices was already described), as well as information about the CAMSAP plasmids and antibodies used in this newly-revised version.

Reviewer #3 (Comments to the Authors (Required)):

The authors nicely responded to the reviewers' comments and have conducted additional analysis and experiments, most importantly to address a major concern about the potential role of CAMSAP proteins (or other components of the basal process varicosities) in regulating microtubule growth. It is great that the authors conducted CAMSAP1 and CAMSAP2 loss-of-function experiments; unfortunately, however, these new experiments do not provide insight into the potential mechanism governing microtubule growth and polarity in the basal process. Thus, the manuscript remains largely descriptive with a model based on correlative experiments.

Overall, there is a bit of a disconnect between the descriptive and functional analysis of the relationship between CAMSAP proteins and EB3 comets (descriptive correlation being done with CAMSAP3 and functional analysis with CAMSAP1 and CAMSAP2). Findings from different groups suggest that the CAMSAP proteins, while related, are likely to play distinct roles in developing neurons, so it would be best to not treat these proteins as interchangeable. The authors state that CAMSAP1 and CAMSAP2 are the predominant CAMSAP proteins expressed in the RG cells (lines 169-170), but they analyze the co-localization of CAMSAP3 with EB3 comets. If the authors feel that CAMSAP1 and CAMSAP2 are the main players, then it would make sense to analyze the relative localization of these two CAMSAP proteins with EB3 comets.
We have now analyzed the localization of CAMSAP1 & 2 using constructs obtained from the Akhmanova lab (Jiang et al, 2014, Dev. Cell). These two factors were observed to robustly localize to swelling (Fig. 4C-E). CAMSAP1 was particularly abundant at the edges of the varicosities, though it could also be observed inside. EB3-mcherry could be seen budding from these CAMSAP foci (Fig. 4G). Live signals for CAMSAP1 and 2, as well as for EB3-mcherry, were extremely low and we did not manage to perform a quantitative assessment of colocalization. We however note that both EB3 comets and CAMSAP1 & 2 concentrate in basal process swellings.

Conversely, the authors did not analyze whether the loss of CAMSAP3 has any effect on EB3 comets or microtubules growth and polarity. Even though CAMSAP3 expression may be lower, it might still play an important role in microtubule dynamics and organization (e.g. Pongrakhananon et al. 2018, PMID 30190432).
We agree but, given its low expression and the results obtained for CAMSAP1 & 2 localization and loss of function, we have not performed this experiment.

Minor comments:

1. The significance of the varicosities is also still unclear. There is a correlation between EB3 comet initiation and CAMSAP3 (and GalNacT2) in the shaft, but this correlation is much stronger in the varicosities, which suggests that CAMSAP3 (or CAMSAP1/2) alone is not sufficient.
We indeed do not know whether CAMSAPs are sufficient for this process, but we now show they are (at least partially) required.

2. The over-expression of CAMSAP3 does not necessarily equate to CAMSAP3 gain-of-function, but it would be helpful for the authors to comment on whether EB3 comet behavior is similar between neurons expressing and not expressing CAMSAP3-GFP. Also, it would be helpful to see the expression/distribution of endogenous CAMPSAP3.
We have not identified any significant difference in EB3 comet formation rates in CAMSAP3-expressing cells vs non-expressing cells (0,134 vs 0.128 comets/min/varicosity), which is now mentioned in the text.

3. Regarding naming different parts of the basal process (e.g. Figure 1F): Another reviewer commented on the different usages of basal, apical, etc. One thought: Would labeling the three sections of the basal process as "distal," "medial," and "proximal" be preferable to basal, medial, apical? This isn't a major concern.
Yes, indeed this is better and has been changed accordingly.

4. In Supplemental Figure 2B: Please specify that CAMSAP1/2 is shown in B&W channel.
This might indeed have been confusing, and has been modified.

April 9, 2021

RE: JCB Manuscript #202003151RR

Dr. Alexandre D Baffet
Institut Curie, PSL Research University, CNRS UMR144, Paris, France
12, rue Lhomond
Paris 75005
France

Dear Dr. Baffet:

Thank you for submitting your revised manuscript entitled "CAMSAPs organize an acentrosomal microtubule network from basal varicosities in radial glial cells". We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

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