Centrosome-mediated microtubule remodeling during axon formation in human iPSC-derived neurons

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Dear Casper,

Thanks for submitting your manuscript to The EMBO Journal. This submission comes via Review Commons where the review process was carried out. I was also the handling editor at Review Commons. I have now had a chance to take a look at the submission for The EMBO Journal and looked at referee comments and your point-by-point response.

I find the analysis interesting and timely, and appreciate the model system used to look at the role of centrosomes during axon formation. I also recognise the concerns raised by the referees and in particular referee #1, but also appreciate your response to the concerns. It will be important to add the requested controls and to better correlate the reported findings with the centriole number. I also hope your proposal to come up with better tools to deplete centrioles will work as this will clearly strengthen the analysis.

Therefore, should you be able to address the concerns raised by the referees as outlined then I would like to invite you to submit a revised manuscript. Let me know if we need to discuss anything further - happy to do so!

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website.

I thank you for the opportunity to consider your work for publication. I look forward to your revision.

with best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Review #1

Evidence, reproducibility and clarity (Required)

The manuscript "Centrosome-mediated microtubule remodeling during axon formation in human iPSC-derived neurons" by Lindhout et al. is a timely study addressing the role of the centrosome, as main microtubule organizer, in axon formation during the transition from proliferating neural stem cells to differentiating neurons. Previous work has shown that during this transition centrosomal microtubule organizing activity is downregulated and that non-centrosomal microtubule organization contributes to the differentiation of neurons. However, to what extent centrosomes still play a role in the early stages of differentiation including axon specification and growth has remained controversial. Since culture of differentiating embryonic rodent neurons, a widely used standard model, does not allow analysis of the very early stages when centrosomes are still active, the authors have employed human iPSCs. These were treated with the PLK4 inhibitor centrinone B to eliminate centrioles (and consequently centrosomes) and differentiated into neurons. The authors found that centrinone B-treated neurons displayed various axonal defects at later differentiation stages such as immature action potentials, lack of axon initial segment-localized TRIM46 protein, and altered growth cone morphologies. They further show that axon-specific, plus-end out microtubule organization is impaired after centrinone B treatment and propose that this interferes with proper axon development and function. Overall I consider this a very timely and important study, designed to clarify a long-standing dispute about the role of centrosomes during neuronal differentiation and more specifically for axon specification and growth. Unfortunately, several conclusions are based on limited experimental support and crucial experimental controls are lacking. While there are many interesting observations, some fundamental questions that the authors set out to answer were not addressed. **Major points:** 1) A major issue is that most experiments are based on comparing centrinone B-treated cells with untreated control cells. However, rather than comparing conditions with/without drug, all experiments should compare centrosome-lacking with centrosome-containing cells. This is important to eliminate the possibility of unspecific effects of the drug treatment and of specific effects of PLK4 inhibition that are unrelated to centriole maintenance. Related to this: I could not find a clear description of how centrinone B treatment was done (when was the drug added and for how long? Were neurons kept in centrinone B until analysis?) Another important control would be to add centrinone B immediately after cells initiate neuronal differentiation (which will not affect centriole number) to confirm that no defects are observed in this condition. 2) Only about 50%
of cells lack both centrioles after centrinone B treatment. However, even in the few experiments that relate phenotypes to centriole number (see point 1), the authors only compare cells with 2 vs <=1 centrioles (Fig 2d,e,f). However, a single remaining centriole may be sufficient to organize PCM and function as centrosome. So technically, in these experiments there is no group quantified, in which all cells lack a centrosome. Moreover, the quantifications lack a group that was not treated with centrinone B. 3) One of the most important questions, whether cells lacking a centrosome (0 centrioles) display any axon specification or growth defects, has been addressed only by Trim46/AnkG staining in Fig. 2. Additional analyses and quantifications would be useful to clarify how centrosome loss affects neuronal differentiation: are axons always specified? Is there a single axon? Do axons grow properly? Are any observed defects temporary or also observed at later stages? 4) The gradual decline in centrosomal gamma-tubulin signal in differentiating neurons (Fig. 1b,c) has been observed several times in various previous publications and as such is not a major finding. Since the authors aim to study the role of centrosomal microtubule organization in neuronal differentiation, they should look at centrosomal microtubules not just gamma-tubulin. Up to what stage is the centrosome active as MTOC? Does MTOC function correlate with centrosomal gamma-tubulin levels? Currently, the heading and conclusions of the first results paragraph are not supported by the data (see also discussion). 5) Since in centrinone B-treated cells TRIM46 is reduced at centrosomes but also fails to accumulate in AnkG positive processes, an important control would be to check total expression levels of TRIM46 by westernblot. 6) The statistical information in the legends is confusing: for all the graphs two n-values are given, 'N' and 'n'. The authors should state only one n-value, the one used for statistical testing. The other number should be stated but not referred to as 'n' or 'N'. 7) The microtubule polarity data in Fig.4 and related suppl. data is very hard to digest. I suggest to present this differently. The reader needs to be guided better and some data may be removed. Overall the observed defects seem relatively mild and in some cases where the text claims an effect the figure shows 'ns' - no significant changes (e.g. 4C.D.E.F). **Minor points:** 1) Introduction: centrosomes do not 'transform into cilia'. Cilia are entirely different structures with basal bodies at their base, please rephrase. 2) The STED data on the sub-centrosomal distribution of TRIM46 is of poor quality. It appears to provide hardly more information than the confocal imaging. In particular in the case of gamma-tubulin there does not seem to be any resolution gain, which is odd, considering that even SIM provides better resolution than what is seen in the images. 3) 1F and 2C: The staining of Trim46 at centrioles looks very different. In 2C there does not seem to be any significant downregulation. 4) It was not clear to me why the proteomic analysis did not indicate the early differentiation phenotype that the authors observed after centrinone B treatment?

**Significance (Required)**

To my knowledge this work is the first that is centered on molecular aspects of microtubule network remodeling, in particular the role of centrosomes, during neurogenesis in vitro, using iPSCs rather than primary embryonic neuron culture. I believe this system has great potential and is the system of choice for addressing these types of questions. Despite this conceptual/technical advance, the work falls a bit short in providing a careful characterization of the state of the microtubule network at the different stages and also lacks essential controls. This is a major issue since there are no comparable studies and a conceptual framework first needs to be established. Target audience are cell biologists interested in the centrosome and microtubules and their roles in development and neurobiologist interested in the cell biology underlying neuronal differentiation. My own expertise is in centrosome and microtubule cell biology including in differentiated cells and therefore is well suited to evaluate the presented study. REFEREES CROSS-COMMENTING The other two reviewers are clearly more positive than me, but their comments do not change my critical view of this manuscript. Reviewer #1 mentioned PLK4 RNAi as a control for the centrinone B treatment. However, this does not rule out
that inhibition of the kinase PLK4 may affect neuronal differentiation independent of centriole elimination. As I stated in my review, the authors need to relate their phenotypes to the absence/presence of centrioles, not drug treatment and include some important, additional control. Without this, I feel that this paper will raise more questions and not settle a controversial issue, as intended.

**Review #2**

**Evidence, reproducibility and clarity (Required)**

In this manuscript by Lindhout and colleagues, centrosome-mediated microtubules (MTs) remodeling is investigated using, predominantly, human iPSC-derived neurons. They exploit live-cell imaging, electrophysiology and mass spectrometry to investigate the role of centrosomes during de novo axon development (and not regeneration, as it is commonly done in the rodent system using primary neurons). They demonstrate that MT centrosomes are essential during axon specification by organizing MT. This function dominates during early stages in neuronal morphogenesis and is gradually lost. This is a very nice paper, clear in its significance and presentation. I really enjoyed reading it, as it addresses complex and fundamental cell biological questions very beautifully and in a highly concise manner. I only have relatively minor points that may be addressed to improve this interesting manuscript. **Specific comments** Figure 1B: I recommend showing more channels in isolation, as these demonstrate and characterize the system as a whole. It seems that the Centrinone inhibitor is relatively specific in its action to inhibit PLKs. But would it be possible, in addition to pharmacological approaches, to undertake shRNA experiments? I would also like to encourage the authors to include measurements of axon length and, possibly, branching. It increases the significance of the results and also firmly establishes the human neuron system presented. Figure 4D may be labelled incorrectly. Distal should be proximal.

**Significance (Required)**

This is a beautiful study that studies very convincingly the function of centrosomes during neuronal polarisation on microtubule polarity in human neurons. It is exceedingly well done, uses a number of advanced techniques.
Evidence, reproducibility and clarity (Required)

This paper by Lindhout et al investigates the role of the centrosome in axon formation in human neurons. The authors observe that TRIM46 localizes to centrosome-associated structures in human cells at early stages, followed by a redistribution to the axon initial segment at later stages. Depletion of centrioles using the Centrinone B compound shows that centrosomes are required for TRIM46 targeting to the axon initial segment. The authors then investigate how centriole depletion affects axon development and find immature action potential firing, altered expression of growth cone proteins, altered growth cone morphology, and altered organization of axonal microtubules. This is a well-performed study on the role of centrosomes in axon development in human neurons. The text is clearly written and the figures are well organized. The mechanistic link between the observations on the transient localization of TRIM46 to centrosomes and the observations on axon development could have been strengthened as it is not clear how these are linked, but I do not think that this is required for this manuscript. **Major comments**

1. The authors investigate action potential firing in Centrinone B-treated neurons (Figure 2), but the underlying rationale is not entirely clear. The authors show that AnkG distribution at the axon initial segment is not affected in Centrinone B-treated neurons, but that TRIM46 localization there is reduced. The same group previously showed that TRIM46 knockdown (Van Beuningen et al., Neuron 2015) causes a mild decrease of the percentage neurons with an axon initial segment positive for a pan-sodium channel marker. The authors should investigate sodium channel distribution in the axon initial segment of Centrinone B-treated neurons to determine whether this can be related to the effects on action potential firing in these neurons. **Minor comments**

1. The magenta and red color combinations in figure panels 1B and 2C are hard to distinguish, a different combination of colors would work better for visualization.
2."In addition, neurons treated with Centrinone-B did not display a progressive maturation of AP properties from day 10 to day 14, as was observed in control neurons (FIG 2I)." - the difference between control and Centrinone B-treated neurons does not seem to be as great as the authors suggest. Can this data be quantified to support the statement that progressive maturation of AP properties does not occur following centrinone B treatment? Alternatively, the text could be reworded.
3. The authors make a strong case for studying de novo polarization of axons in human neurons in the introduction. In their analysis, did they observe any phenotypes that appear uniquely human (in addition to TRIM46 localization to centrosomes) or are the findings consistent with
Significance (Required)

This study investigates the role of centrosomes in axon development in human neurons, in which de novo axon polarisation occurs. The findings are therefore of interest in light of the existing literature on fly and rodent neurons and will appeal to an audience of cell biologists and molecular neuroscientists. REFEREES CROSS-COMMENTING I read the review reports and agree with most points raised by the other reviewers. Reviewer 1 raises some important points on centrinone B treatment controls that I think the authors should try to address. As reviewer 2 also points out, the study relies on a single drug so a complimentary approach might be an alternative. I also had difficulty grasping Figure 4 so reorganising this and rewording the text would be helpful.
The manuscript "Centrosome-mediated microtubule remodeling during axon formation in human iPSC-derived neurons" by Lindhout et al. is a timely study addressing the role of the centrosome, as main microtubule organizer, in axon formation during the transition from proliferating neural stem cells to differentiating neurons. Previous work has shown that during this transition centrosomal microtubule organizing activity is downregulated and that non-centrosomal microtubule organization contributes to the differentiation of neurons. However, to what extent centrosomes still play a role in the early stages of differentiation including axon specification and growth has remained controversial. Since culture of differentiating embryonic rodent neurons, a widely used standard model, does not allow analysis of the very early stages when centrosomes are still active, the authors have employed human iPSCs. These were treated with the PLK4 inhibitor centrinone B to eliminate centrioles (and consequently centrosomes) and differentiated into neurons. The authors found that centrinone B-treated neurons displayed various axonal defects at later differentiation stages such as immature action potentials, lack of axon initial segment-localized TRIM46 protein, and altered growth cone morphologies. They further show that axon-specific, plus-end out microtubule organization is impaired after centrinone B treatment and propose that this interferes with proper axon development and function. Overall I consider this a very timely and important study, designed to clarify a long-standing dispute about the role of centrosomes during neuronal differentiation and more specifically for axon specification and growth. Unfortunately, several conclusions are based on limited experimental support and crucial experimental controls are lacking. While there are many interesting observations, some fundamental questions that the authors set out to answer were not addressed.

We thank the reviewer for appreciating the timely topic as well as the observations presented in this study. We added new experiments and applied textual to address the comments of the reviewer in a revised version of the manuscript.

**Major points:**

1) A major issue is that most experiments are based on comparing centrinone B-treated cells with untreated control cells. However, rather than comparing conditions with/without drug, all experiments should compare centrosome-lacking with centrosome-containing cells. This is important to eliminate the possibility of unspecific effects of the drug treatment and of specific effects of PLK4 inhibition that are unrelated to centriole maintenance. Related to this: I could not find a clear description of how centrinone B treatment was done (when was the drug added and for how long? Were neurons kept in centrinone B until analysis?)

Another important control would be to add centrinone B immediately after cells initiate neuronal differentiation (which will not affect centriole number) to confirm that no defects are observed in this condition.
We thank the reviewer for raising this point and for suggesting these control experiments. The reviewer is correct in that this study, as well as many other recent studies, used the PLK4 inhibitor Centrinone-B as a primary tool to study centrosome dysfunction (Yeow Z. H. et al., Nature 2020; Chinen T. et al., EMBO J 2020; Mercey O. et al., Nature Cell Biology 2019; Luo Y. et al., Nature Communications 2019; avilan M.P. et al., EMBO J 2018). We agree with the reviewer that it is important to control for possible off-target effects as well as PLK4 inhibition effects unrelated to centriole maintenance, and added a series of control experiments to validate our findings. We repeated our key experiments with the suggested control condition in which cells were treated with Centrinone-B after neuronal differentiation, which therefore will not affect centriole number. We observed no significant differences between untreated and post-differentiation Centrinone-B treatment for our key observations, including axonal Trim46 targeting (FIG S2L,M), AP amplitude and half-width (FIG S2R,S) and the axonal microtubule organization (FIG S4S,T,U), thereby validating the our key findings.

We also attempted to correlate more findings with centriole numbers in cells, however these experiments have proven to be technically challenging. For many of the findings presented in this study it was not feasible to perform such experiments, as the required fixation method to visualize individual centrioles was not compatible with most of the experimental set-ups. For the live-cell imaging experiments we aimed to correlate our observations with centriole numbers by performing post-hoc analysis, which involved culturing neurons on grid coverslips for the microtubule dynamic experiments or using a dye in the internal pipette solution for the electrophysiology experiments. This was technically challenging as human iPSC-derived neuron cultures are quite delicate and any subtle changes in protocols or additional handlings can be critical. Unfortunately, the neurons did not grow well on the grid coverslips, despite different trials of pre-coating coverslip treatments, and also the patched neurons did not recover well enough after electrophysiological recordings for post-hoc analysis experiments.

As an alternative approach and in addition to the suggested experiments, we set out a POC study to design new tools to deplete centrioles based on genetic manipulation of different centriole duplication regulators. With this study we aimed to provide alternatives for Centrinone-B to confirm the key findings in this study, as well as to provide additional tools for future centrosome studies. We designed eight different lentiviral CRISPR/Cas9 knock-out constructs targeting human SAS6, STIL and CENPJ, all regulators of centriole duplication, and observed ~95% infection efficiency of all viral constructs in our cultures. Determining centriole numbers in neuronal stem cells for each construct revealed that hSAS6 gRNA#1 had the most profound effect on centriole loss (FIG S2G,H). For full transparency we also share the results and sequences of the other constructs, which had less profound effects on centriole number (see data below). We repeated our key finding on perturbed axonal Trim46 targeting using CRISPR/Cas9 hSas6 gRNA#1 and found a significant reduction in Trim46 appearance at axons, thereby excluding possible off-target as well as PLK4 inhibition effects unrelated to centriole number (FIG S2L,J).

As for the description of the Centrinone-B treatment, the paragraph Pharmacological Treatments in the Material & Methods section stated the following: “For centriole loss experiments, cells were treated with Centrinone-B (500 nM; HY-18683, MedChemExpress) 1 hour after plating and treatment was continued with every following medium change to prevent unintentional wash-out.” The days on which medium changes are performed can be found in the paragraph Human iPSC-derived culture of the Material & Methods Section.
2) Only about 50% of cells lack both centrioles after centrinone B treatment. However, even in the few experiments that relate phenotypes to centriole number (see point 1), the authors only compare cells with 2 vs <=1 centrioles (Fig 2d,e,f). However, a single remaining centriole may be sufficient to organize PCM and function as centrosome. So technically, in these experiments there is no group quantified, in which all cells lack a centrosome. Moreover, the quantifications lack a group that was not treated with centrinone B.

The data of cells containing <=1 centriole(s) is pooled in FIG 2D-F, as the phenotype of mistargeted axonal Trim46 was consistently observed in cells lacking either 1 or 2 centrioles. This observation suggests that correct targeting of axonal Trim46 requires fully intact centrosomes containing 2 centrioles, as the presence of 1 centriole is not sufficient to mediate axonal Trim46 targeting. We agree with the reviewer that 1 centriole may still execute centrosome functions, although likely to a lesser extent. It is in our view not uncommon in biology to find phenotypes with only a partial perturbation of a subcellular structure. We agree with the reviewer that it is relevant to also show the data on the specific centriole numbers and to include an untreated group. We have now extended the dataset of our key finding showing a correlation of centriole loss and mistargeted axonal Trim46 with these additional measurements in a revised version of the manuscript (FIG S2F).

3) One of the most important questions, whether cells lacking a centrosome (0 centrioles) display any axon specification or growth defects, has been addressed only by Trim46/AnkG staining in Fig. 2. Additional analyses and quantifications would be useful to clarify how centrosome loss affects neuronal differentiation: are axons always specified? Is there a single axon? Do axons grow properly? Are any observed defects temporary or also observed at later stages?

We thank the reviewer for raising these questions. In addition to the observed effects of centriole removal on AIS assembly (FIG 2C-F), we also reported numerous other observations related to axon development and growth. Specifically, we found that centriole removal resulted in delayed action potential maturation (FIG 2H-N, S2N-Q), suppressed expression of growth cone proteins (FIG 3A-C, S3A-C), impaired growth cone morphologies (FIG 3D-H, S3E) and perturbed axon-specific microtubule remodeling (FIG 4A-R, S4A-R). As for the temporary nature of the defects, the majority of experiments were performed at different timepoints to gain insights in the dynamics of the identified
defects during development. Our findings indicated that the defects became overall more apparent at later stages, as was the case for delayed action potential maturation (FIG 2H-N, S2N-Q), suppressed expression of growth cone proteins (FIG 3A-C, S3A-C) and perturbed axon-specific microtubule remodeling (FIG 4A-R, S4A-R). Contrarily, the effects on growth cone morphologies (FIG 3D-H, S3E) were most profound at early time points. Together, these data may imply a specific sequence of events of axon developmental defects upon centriole loss. At later stages (day 14, stage 3), we consistently observed in all conditions that neurons contained a single neurite that has grown at least twice as long as the other neurites, indicating successful specification of a single axon that is unaffected by centriole loss. We agree with the reviewer that it is relevant to investigate the effect of centriole removal on outgrowth. To address this, we quantified the length and number of primary branches at day 5, which represents the latest time point in which the lengthy neurites can still reliably be traced in our system. At this stage, most neurons have not formed axons yet and we observed no differences in neurite length and number (see data below).

Response Figure 2

4) The gradual decline in centrosomal gamma-tubulin signal in differentiating neurons (Fig. 1b,c) has been observed several times in various previous publications and as such is not a major finding. Since the authors aim to study the role of centrosomal microtubule organization in neuronal differentiation, they should look at centrosomal microtubules not just gamma-tubulin. Up to what stage is the centrosome active as MTOC? Does MTOC function correlate with centrosomal gamma-tubulin levels? Currently, the heading and conclusions of the first results paragraph are not supported by the data (see also discussion).

The reviewer is correct in that the gradual decline in centrosomal gamma-tubulin has previously been reported for differentiating non-human neurons. To the best of our knowledge, however, this has not been investigated for human neurons. Therefore we considered it to be important to validate this finding in our human neuron model system and stated our observation as followed in the Results section: “We found that the γ-Tubulin levels at centrosomes were consistently high in stage 1 and stage 2 neurons, and markedly reduced by ~50% in stage 3 neurons, consistent with previous findings in dissociated rat neurons (FIG 1A-C) (Stiess et al, 2010).” We have applied textual changes to the Introduction section to state this more clearly here as well. We thank the reviewer for the relevant suggestion to further investigate the role of centrosomes as MTOC at different neurodevelopmental stages. To address this, we performed high resolution 3D STED imaging to resolve the dense microtubule network in neurons at different time points. We observed a shift from a radial towards a non-radial microtubule network as neurons proceed through the early developmental stages 1-3, further indicating a gradual decline of the microtubule-organizing functions of centrosomes correlating with the process of axon development (FIG 1D).
5) Since in centrinone B-treated cells TRIM46 is reduced at centrosomes but also fails to accumulate in AnkG positive processes, an important control would be to check total expression levels of TRIM46 by western blot.

*We thank the reviewer for this suggestion. We conducted a Western Blot experiment to evaluate the Trim46 expression levels and observed no obvious difference between control and Centrinone-B treated neurons (FIG S2K).*

6) The statistical information in the legends is confusing: for all the graphs two n-values are given, 'N' and 'n'. The authors should state only one n-value, the one used for statistical testing. The other number should be stated but not referred to as 'n' or 'N'.

*We thank the reviewer for pointing this out. We have applied the requested textual changes in a revised version of the manuscript to better clarify this.*

7) The microtubule polarity data in Fig.4 and related suppl. data is very hard to digest. I suggest to present this differently. The reader needs to be guided better and some data may be removed. Overall the observed defects seem relatively mild and in some cases where the text claims an effect the figure shows "ns" - no significant changes (e.g. 4C,D,E,F).

*We thank the reviewer for raising this point. We have critically reviewed and adapted the representation of the data in Figure 4 and Supplementary Figure 4 as well as the corresponding description of the findings in the Results section in a revised version of the manuscript. The key finding of this figure is that the organization of the axon-specific microtubule network, characterized by parallel plus-end out microtubule bundles, is perturbed in developing Centrinone-B treated neurons. This is most strikingly observed in FIG 4O, which shows that only ~50% of the axons of Centrinone-B treated neurons have developed an axon-specific microtubule network whereas this is ~90% in control neurons. We thank the reviewer for the suggestion to re-evaluate the data in FIG 4C-F. We have now increased the statistical group number n for the data presented in FIG 4C-E, 4F-G, S4B-E, and S4G-J. Consistent with FIG 4O, we now also observe a significant ~50% reduction of neurons with a uniform plus-end out microtubule organization in axons upon Centrinone-B treatment when measuring only the dynamic microtubule population (FIG 4F).*

**Minor points:**

1) Introduction: centrosomes do not 'transform into cilia'. Cilia are entirely different structures with basal bodies at their base, please rephrase.

*We thank the reviewer for pointing this out and have rephrased the text in the Introduction in a revised version of the manuscript.*

2) The STED data on the sub-centrosomal distribution of TRIM46 is of poor quality. It appears to provide hardly more information than the confocal imaging. In particular in the case of gamma-tubulin there does not seem to be any resolution gain, which is odd, considering that even SIM provides better resolution than what is seen in the images.

*The image of gamma-tubulin in FIG 1F is obtained by confocal microscopy whereas Trim46 and Centrin images were obtained STED, thereby explaining the non-apparent resolution gain for gamma-tubulin. This was also described in the figure legend: “Centrosomes of human iPSC-derived NSCs (day*
0) with STED imaging of Trim46 and Centrin immunostaining, and confocal imaging of γ-Tubulin immunostaining.” We applied textual changes in the Results section to further clarify this. The aim of this STED experiment was to better resolve the centrosome-associated Trim46 structures and to correlate its localization to known centrosomal substructures. Resolving the single oval Trim46 structure using STED revealed that it is composed of a cloud of multiple small puncta that does not appear to fully co-localize with centrioles nor the PCM.

3) 1F and 2C: The staining of Trim46 at centrioles looks very different. In 2C there does not seem to be any significant downregulation.

We thank the reviewer for pointing this out. The confocal microscopy images of Trim46 structures in FIG 1G (previously FIG 1F) and FIG 2C were obtained using different acquisition settings that were optimized for the respective experiment. In FIG 1G we used the same image acquisition settings for stage 2 and stage 3 neurons to directly compare the Trim46 levels at centrosomes between these stages. In FIG 2C higher exposure time and laser power was used to still visualize Trim46 structures at centrosomes, as this enabled us to better identify centrosomes as these were positive for both Centrin and Trim46. Hence a direct comparison of Trim46 fluorescence intensity levels between FIG 1G and FIG 2C cannot be made.

4) It was not clear to me why the proteomic analysis did not indicate the early differentiation phenotype that the authors observed after centrinone B treatment?

We thank the reviewer for raising this point. The proteomic data represents the ratios of expression profiles of different time points. Specifically, it represents the ratio of samples taken at day 3 or day 7 over samples taken at day 1. This means day 1 is set as a calibration point, however, the premature neuronal differentiation phenotype is already apparent at day 1 and this phenotype is therefore not reflected in this analysis. We have now applied textual changes to the figure legend to better clarify this.

Reviewer #1 (Significance (Required)):

To my knowledge this work is the first that is centered on molecular aspects of microtubule network remodeling, in particular the role of centrosomes, during neurogenesis in vitro, using iPSCs rather than primary embryonic neuron culture. I believe this system has great potential and is the system of choice for addressing these types of questions. Despite this conceptual/technical advance, the work falls a bit short in providing a careful characterization of the state of the microtubule network at the different stages and also lacks essential controls. This is a major issue since there are no comparable studies and a conceptual framework first needs to be established. Target audience are cell biologists interested in the centrosome and microtubules and their roles in development and neurobiologist interested in the cell biology underlying neuronal differentiation. My own expertise is in centrosome and microtubule cell biology including in differentiated cells and therefore is well suited to evaluate the presented study.

We thank the reviewer for appreciating the potential of human iPSC-derived neurons as a model system for the questions addressed here. We are pleased to have received the comments of the reviewer and added new control experiments and applied textual changes to address these in a revised manuscript.
REFEREES CROSS-COMMENTING

The other two reviewers are clearly more positive than me, but their comments do not change my critical view of this manuscript. Reviewer #1 mentioned PLK4 RNAi as a control for the centrinone B treatment. However, this does not rule out that inhibition of the kinase PLK4 may affect neuronal differentiation independent of centriole elimination. As I stated in my review, the authors need to relate their phenotypes to the absence/presence of centrioles, not drug treatment and include some important, additional control. Without this, I feel that this paper will raise more questions and not settle a controversial issue, as intended.

*We thank the reviewer for acknowledging the divergence in the review rapport. We agree with the reviewer that PLK4 RNAi would not serve as the optimal control experiment here and we therefore included the series of control experiments as described above.*

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

In this manuscript by Lindhout and colleagues, centrosome-mediated microtubules (MTs) remodeling is investigated using, predominantly, human iPSC-derived neurons. They exploit live-cell imaging, electrophysiology and mass spectrometry to investigate the role of centrosomes during de novo axon development (and not regeneration, as it is commonly done in the rodent system using primary neurons). They demonstrate that MT centrosomes are essential during axon specification by organizing MT. This function dominates during early stages in neuronal morphogenesis and is gradually lost. This is a very nice paper, clear in its significance and presentation. I really enjoyed reading it, as it addresses complex and fundamental cell biological questions very beautifully and in a highly concise manner. I only have relatively minor points that may be addressed to improve this interesting manuscript.

*We are very pleased that the reviewer enjoyed reading the paper and that the significance as well as the presentation of the study is much appreciated. We added new experiments to address the comments of the reviewer in a revised version of the manuscript.*

1) Figure 1B: I recommend showing more channels in isolation, as these demonstrate and characterize the system as a whole.

*We thank the reviewer for this suggestion. We have isolated the channels in FIG 1B in a revised version of the manuscript.*

2) It seems that the Centrinone inhibitor is relatively specific in its action to inhibit PLKs. But would it be possible, in addition to pharmacological approaches, to undertake shRNA experiments?

*We thank the reviewer for raising this point and we agree that it is relevant to include more control experiments to further validate our findings. Indeed, Centrinone-B seems to target PLK4 relatively specifically and therefore we have set-out alternative strategies for control experiments other than shRNA depletion, as also suggested by reviewer #1. We have performed an exploratory pilot study to*
design new tools to deplete centrioles, which is based on genetic manipulation of other regulatory factors for centriole duplication (see Response Figure 1 above). We continued with lentiviral CRISPR/Cas9 hSAS6 gRNA#1 construct, as this construct had the most profound effect on centriole loss (FIG S2G,H). Similar as Centrinone-B treatment, we also observed significant reduction in Trim46 targeting at axons upon lentiviral infection with CRISPR/Cas9 hSAS6 gRNA#1, thereby confirming our key finding (FIG S2I,J). In addition, we also included a control condition for several datasets in which cells were treated with Centrinone-B after neuronal differentiation, thereby controlling for the possible effects of PLK4 that are unrelated to centriole maintenance in postmitotic cells. Unlike regular Centrinone-B treatment, no significant differences with late post-differentiation Centrinone-B treatment were found for axonal Trim46 targeting (FIG S2L,M), AP amplitude and half-width (FIG S2R,S) and the axonal microtubule organization (FIG S4S,T,U), thereby further validating the findings presented in this study.

3) I would also like to encourage the authors to include measurements of axon length and, possibly, branching. It increases the significance of the results and also firmly establishes the human neuron system presented.

We thank the reviewer for this suggestion. A remarkable observation that we made was that human axons tend to grow extremely long already at early stages compared to e.g. rat neurons, and we rarely observed axon branching at the different developmental stages investigated in this study. We measured neurite length and number of human neurons at day 5, when it is still possible to reliably trace neurites in this system, and observed no significant changes at this stage during which most neurons are yet unpolarized (see Response Figure 1 above).

4) Figure 4D may be labelled incorrectly. Distal should be proximal.

We thank the reviewer for catching this discrepancy. This is corrected in a revised version of the manuscript.

Reviewer #2 (Significance (Required)):

This is a beautiful study that studies very convincingly the function of centrosomes during neuronal polarisation on microtubule polarity in human neurons. It is exceedingly well done, uses a number of advanced techniques.

We sincerely thank the reviewer for appreciating the quality of the study.

Reviewer #3 (Evidence, reproducibility and clarity (Required)):

This paper by Lindhout et al investigates the role of the centrosome in axon formation in human neurons. The authors observe that TRIM46 localizes to centrosome-associated structures in human cells at early stages, followed by a redistribution to the axon initial segment at later stages. Depletion of centrioles using the Centrinone B compound shows that centrosomes are required for TRIM46 targeting to the axon initial segment. The authors then investigate how centriole depletion affects axon development and find immature action potential firing, altered expression of growth cone proteins, altered growth cone morphology, and altered organization of axonal microtubules. This is a well-performed study on the role of
centrosomes in axon development in human neurons. The text is clearly written and the figures are well organized. The mechanistic link between the observations on the transient localization of TRIM46 to centrosomes and the observations on axon development could have been strengthened as it is not clear how these are linked, but I do not think that this is required for this manuscript.

We thank the reviewer for appreciating how the study is performed as well as its representation. We performed additional experiments and applied textual changes to address the comments of the reviewer in a revised version of the manuscript.

**Major comments**

1) The authors investigate action potential firing in Centrinone B-treated neurons (Figure 2), but the underlying rationale is not entirely clear. The authors show that AnkG distribution at the axon initial segment is not affected in Centrinone B-treated neurons, but that TRIM46 localization there is reduced. The same group previously showed that TRIM46 knockdown (Van Beuningen et al., Neuron 2015) causes a mild decrease of the percentage neurons with an axon initial segment positive for a pan-sodium channel marker. The authors should investigate sodium channel distribution in the axon initial segment of Centrinone B-treated neurons to determine whether this can be related to the effects on action potential firing in these neurons.

We thank the reviewer for raising this point. Indeed, a previous publication from our group showed mild effects of Trim46 depletion on targeting of sodium channels (van Beuningen et al., 2015). In this study we also report a significantly smaller maximum sodium current for Centrinone-B treated neurons (FIG 2N). Overall, human iPSC-derived neurons have much slower sodium peaks compared to recordings performed in e.g. neurons of mouse slices. This suggests that at these early stages of development, the amount of sodium channels in control conditions is already considerably low. This is also shown by the slow speed and low amplitudes of action potentials when compared to e.g. more mature rodent neurons of the same age. We have attempted to measure the amount and distribution of these low levels of sodium channels using immunofluorescence (see Response Figure 3 below). We found no significant change in distribution and amount of sodium channels between control and Centrinone-B treatment: the sodium channels distributed across similar lengths in the developing axon (Response Figure 3A), and seemed distributed evenly throughout these stretches based on average fluorescence intensity (Response Figure 3B). A small trend towards a decrease in sodium channel signal is observed in Centrinone-B treated neurons. However, our approach to visualizing sodium channel clustering in immunofluorescence does not appear sufficient to detect differences on these low levels. While more advanced approaches using superresolution or CRISPR knock-ins could be employed, we believe that detecting these differences using electrophysiology is an accurate method to observe these differences.
**Minor comments**

1) The magenta and red color combinations in figure panels 1B and 2C are hard to distinguish, a different combination of colors would work better for visualization. 

*We thank the reviewer for this suggestion. We have now also added isolated channels for these figures for better visualization in a revised version of the manuscript.*

2) "In addition, neurons treated with Centrinone-B did not display a progressive maturation of AP properties from day 10 to day 14, as was observed in control neurons (FIG 2I)." - the difference between control and Centrinone B-treated neurons does not seem to be as great as the authors suggest. Can this data be quantified to support the statement that progressive maturation of AP properties does not occur following centrinone B treatment? Alternatively, the text could be reworded.

*We thank the reviewer for raising this point. The data on AP amplitude and AP half-width shown in FIG 2J (previously FIG 2I) are also shown and statistically tested in FIG S2N and FIG S2O, respectively, which altogether formed the basis for this conclusion. Both the AP amplitude as well as the AP half-width showed progressive maturation over time in control neurons, reflected by a significant increase in AP amplitude and a significant increase in AP half-width, but did not in Centrinone-B treated neurons (FIG S2N,O). Consistently, there was a significant difference in AP amplitude as well as AP half-width in mature control neurons at day 13-14 compared to Centrinone-B treated neurons at the same time points (FIG S2N,O). We have now better referred to FIG S2N,O when drawing this conclusion in the Results section in a revised version of the manuscript.*

3) The authors make a strong case for studying de novo polarization of axons in human neurons in the introduction. In their analysis, did they observe any phenotypes that appear uniquely human (in addition to TRIM46 localization to centrosomes) or are the findings consistent with observations in other model systems?
We thank the reviewer for bringing this up. Indeed, we have made observations related to axon development in human neurons that were not detected in other non-human model systems before. Of particular interest, we identified a distinct axon developmental stage in human iPSC-derived neurons, marked by an increase in microtubule remodeling and apparent relocation of AIS proteins from the distal (stage 3a) to proximal axon (stage 3b). The transition through the early axon developmental stages 3a and 3b coincided with the time window in which maturation of action potentials occurred. These results are part of a different study which was recently published (Lindhout et al. 2020, doi:10.7554/eLife.58124). To the best of our knowledge, this transition has not been observed in non-human neurons. In the study presented here we also discriminated between proximal and distal axons for the dynamic microtubule remodeling experiments. We observed the most profound differences between control and Centrinone-B treated neurons in distal axons, which can be considered the most mature axonal stage (FIG 4, FIG S4). We have now referred to the Lindhout et al., 2020 study in a revised version of the manuscript.

Reviewer #3 (Significance (Required)):

This study investigates the role of centrosomes in axon development in human neurons, in which de novo axon polarisation occurs. The findings are therefore of interest in light of the existing literature on fly and rodent neurons and will appeal to an audience of cell biologists and molecular neuroscientists.

We thank the reviewer for appreciating the significance of this study.

REFEREES CROSS-COMMENTING

I read the review reports and agree with most points raised by the other reviewers. Reviewer 1 raises some important points on centrinone B treatment controls that I think the authors should try to address. As reviewer 2 also points out, the study relies on a single drug so a complimentary approach might be an alternative. I also had difficulty grasping Figure 4 so reorganising this and rewording the text would be helpful.

We thank the reviewer for these insightful comments. We now conducted additional control experiments to minimize the risk of off-target effects as well as unintentional effects of PLK4 inhibition. We have designed a new tool to deplete centrioles based on genetic manipulation of hSAS6, another important regulator of centriole duplication, as an alternative for the PLK4 inhibitor Centrinone-B (FIG S2G,H). Using this tool, we found a similar effect on mistargeted Trim46 at axons as previously found with Centrinone-B, thereby validating our key finding (FIG S1,J). Additionally, we repeated a large part of the experiments with an extra control condition in which Centrinone-B was added after neuronal differentiation, thereby controlling for PLK4 inhibitory effects unrelated to centriole maintenance. Unlike regular Centrinone-B treatment, no significant differences with late post-differentiation Centrinone-B treatment were found for axonal Trim46 targeting (FIG S2L,M), AP amplitude and half-width (FIG S2R,S) and the axonal microtubule organization (FIG S4S,T,U), thereby further validating the findings presented in this study. The representation of the data as well as the corresponding text of FIG 4 and FIG S4 was critically reviewed and adjusted in a revised version of the manuscript.
Dear Casper,

Thank you for submitting your revised manuscript to The EMBO Journal. Your study has now been seen by the three referees and their comments are provided below.

As you can see below, the three referees appreciate the added changes and support publication here. Referee #1 has a few remaining issues, but they can be addressed with text changes and a better discussion.

When you submit your revised manuscript will you also please take care of the following points

- We need a Data Availability section (place it after the Materials and methods and before Acknowledgements). This is where the mass spec proteomics accession number should be listed

- Please double check that the reference format is correct.

- The supplemental figures should be re-labeled Expanded View Figures (see also our guide to authors). Please also correct the figure callouts in text

- Callouts in the text are missing for Fig EV2Q and EV4K+P

- Each movie file should be zipped together with its legend and the movie legends removed from the main article file. Please also label the movie files MovieEV1 etc

- Please also sort the manuscript sections in the correct order.

- I have asked our publisher to do their pre-publication checks on the paper. They will send me the file within the next few days. Please wait to upload the revised version until you have received their comments.

- We include a synopsis of the paper (see http://emboj.embopress.org/). Please provide me with a general summary statement and 3-5 bullet points that capture the key findings of the paper.

- We also need a summary figure for the synopsis. The size should be 550 wide by [200-400] high (pixels). You can also use something from the figures if that is easier.

Please include a point-by-point response also to the above raised editorial points when you resubmit.

That should be all - let me know if you have any further questions.

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal
Instructions for preparing your revised manuscript:

Please check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
https://bit.ly/EMBOPressFigurePreparationGuideline

IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/authorguide).
- Expanded View files (replacing Supplementary Information)

Please see out instructions to authors
https://www.embopress.org/page/journal/14602075/authorguide#expandedview

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

Further information is available in our Guide For Authors:
https://www.embopress.org/page/journal/14602075/authorguide

The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 28th Apr 2021.

https://emboj.msubmit.net/cgi-bin/main.plex

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Referee #1:

This is a re-review. For a summary and general assessment please refer to my original review.

In the revised manuscript "Centrosome-mediated microtubule remodeling during axon formation in human iPSC-derived neurons" the authors have addressed my concerns by providing additional experiments and by text/figure changes. The manuscript is improved, but there are a few remaining issues. In my opinion, publication would require additional changes to the manuscript.
1) I understand that centriole number counting is not compatible with all the different assays that have been performed. However, the authors claim to analyse the role of the centrosome in neuronal differentiation, so it is essential to show that a centrosome is not present in cells that display phenotypes. As it stands, this is not the case in the majority of cells analysed, since most still contain at least one centriole. The findings are valid, but publication would require addressing these points:

a) text changes in all relevant passages to indicate that centriole loss, rather than centrosome loss, causes the observed phenotypes (title, abstract and main text). The authors correctly refer to centriole loss in most cases where they describe data, but then frequently refer to centrosome or MTOC function when interpreting their results.

b) Mechanistically, the effects of loss of a single centriole may not even be related to the centrosomal MTOC function (since 1 centriole likely maintains MTOC activity; see below). This possibility should be explicitly discussed in the discussion section.

c) the authors argue in their rebuttal letter that a centrosome with a single centriole may be compromised in its MTOC activity. Is this the case here?

d) the remaining centriole after centrinone treatment is expected to be a mother with distal and subdistal appendages and the ability to recruit PCM (gamma-tubulin). In the best of the cases this may reduce MTOC activity by 50% in terms of nucleation, but microtubule anchoring would occur on the mother that carries subdistal appendages (which is expected to be present in most neurons). These are all issues that should be discussed.

2) Fig. 2D: I strongly suggest to display the data of neurons with 0 and neurons with 1 centriole separately. As mentioned in my first review, the authors do not show any experiment, in which phenotypes are analyzed specifically in neurons without a centrosome (no centrioles). This is in contrast to the main message of the study.

3) Considering that only 2 days of centrinone treatment were used, to avoid problems with cell cycle arrest or premature exit, Fig. 2A, B and the description in the text are misleading. They suggest that the authors generated cells, of which up to 50% completely lack centrioles (5 days). First, the claim that there is no significant difference between 2 and 5 day treatments must be a problem with low n (only ~50 cells, 2 experiments). It naturally takes multiple cell cycles with inhibited centriole duplication to completely deplete existing centrioles from a population of cells (e.g. Wong et al. 2015, Science; Lambrus et al., 2016, J Cell Biol). So longer treatment should result in fewer centrioles per cell. Second, after only 2 days of centrinone treatment, only a minority of neurons seems to have only 1 or 0 centrioles (Fig. S2F), but the exact percentages are still not shown. The authors should include in Fig. 2 quantification of centriole number in neurons derived from NPCs after 2 days in centrinone, as used throughout the study (as shown for SAS6 KO in Fig. S2H).

Addressing the above points will help the reader to correctly interpret the results and avoid confusion. In my opinion the technical challenges in this study to produce neurons without centrosome (no centrioles) do not allow to completely settle the controversy about the contribution of the centrosomal MTOC to neuronal differentiation. However, the presented findings are still interesting and relevant, and, with appropriate modifications, would be suitable for publication in EMBO J.
Referee #2:

I believe the authors addressed the main and critical points raised by all reviewers. I support this manuscript to be accepted for publication.

Referee #3:

The authors have convincingly addressed all my concerns in their revision. I support publication of their study in EMBO Journal.
Point-to-point response EMBOJ-2020-106798R “Centrosome-mediated microtubule remodeling during axon formation in human iPSC-derived neurons”

Editorial points

1) We need a Data Availability section (place it after the Materials and methods and before Acknowledgements). This is where the mass spec proteomics accession number should be listed.

_We have now added the Data Availability section covering the MS proteomics accession number._

2) Please double check that the reference format is correct.

_We checked and hereby confirm that the references are in the correct format._

3) The supplemental figures should be re-labeled Expanded View Figures (see also our guide to authors). Please also correct the figure callouts in text

_We have re-labeled the supplemental figures to Expanded View Figures and adjusted figure callouts in text accordingly._

4) Callouts in the text are missing for Fig EV2Q and EV4K+P

_Callouts to these figures have been added in the text in the appropriate sections of the manuscript._

5) Each movie file should be zipped together with its legend and the movie legends removed from the main article file. Please also label the movie files MovieEV1 etc

_The movies have been re-labeled, and movie legends have been moved from the main article file into zip files with corresponding movie files._

6) Please also sort the manuscript sections in the correct order.

_The manuscript sections are now sorted into the correct order._

7) I have asked our publisher to do their pre-publication checks on the paper. They will send me the file within the next few days. Please wait to upload the revised version until you have received their comments.

_Thank you for arranging the pre-publication checks. We have received the comments and addressed these in the re-revised version of the manuscript._

8) We include a synopsis of the paper (see http://emboj.embopress.org/). Please provide me with a general summary statement and 3-5 bullet points that capture the key findings of the paper.

_Standfirst text and bullet points for the synopsis of the paper are now added in a separate manuscript file for resubmission._
9) We also need a summary figure for the synopsis. The size should be 550 wide by [200-400] high (pixels). You can also use something from the figures if that is easier.

We have repurposed the graphical abstract figure into a summary figure for the synopsis.

Referee #1:

This is a re-review. For a summary and general assessment please refer to my original review. In the revised manuscript "Centrosome-mediated microtubule remodeling during axon formation in human iPSC-derived neurons" the authors have addressed my concerns by providing additional experiments and by text/figure changes. The manuscript is improved, but there are a few remaining issues. In my opinion, publication would require additional changes to the manuscript.

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a) text changes in all relevant passages to indicate that centriole loss, rather than centrosome loss, causes the observed phenotypes (title, abstract and main text). The authors correctly refer to centriole loss in most cases where they describe data, but then frequently refer to centrosome or MTOC function when interpreting their results.

b) Mechanistically, the effects of loss of a single centriole may not even be related to the centrosomal MTOC function (since 1 centriole likely maintains MTOC activity; see below). This possibility should be explicitly discussed in the discussion section.

c) the authors argue in their rebuttal letter that a centrosome with a single centriole may be compromised in its MTOC activity. Is this the case here?

d) the remaining centriole after centrinone treatment is expected to be a mother with distal and subdistal appendages and the ability to recruit PCM (gamma-tubulin). In the best of the cases this may reduce MTOC activity by 50% in terms of nucleation, but microtubule anchoring would occur on the mother that carries subdistal appendages (which is expected to be present in most neurons). These are all issues that should be discussed.

We thank the referee for reviewing in great detail the revised version of the manuscript and for appreciating the improvements made. When technically feasible, we correlated experimental observations induced by Centrinone-B treatment with the number of centrioles in cells (FIG 2C-F, FIG S2F). All other effects of Centrinone-B treatment on axon formation were measured in a mixed neuron population of which approximately half of the cells lost at least one centriole. We agree with the reviewer that we cannot exclude that neurons containing one centriole may to some extent still exhibit centrosomal MTOC functions. However, our data clearly shows that developmental axon perturbations are identified in the mixed neuron population generated by Centrinone-B treatment in these datasets, which is indicative of the large effect sizes of the reported findings and also implies that single centriole loss is sufficient to at least partially perturb centrosome functions. In our view, effects measured by centriole loss can be interpreted as effects due to perturbed centrosome functions, since centrioles are the core components of centrosomes and centrioles are, to the best of our knowledge, not known to be
implicated in non-centrosome functions. We made clear textual distinctions throughout the article when describing and interpreting the results. We have now also addressed this concern in the discussion in the re-revised version of the manuscript.

2) Fig. 2D: I strongly suggest to display the data of neurons with 0 and neurons with 1 centriole separately. As mentioned in my first review, the authors do not show any experiment, in which phenotypes are analyzed specifically in neurons without a centrosome (no centrioles). This is in contrast to the main message of the study. We kindly refer the reviewer to FIG S2F where we correlate the data of FIG 2D to centriole numbers, this dataset also includes neurons containing 0 centrioles.

3) Considering that only 2 days of centrinone treatment were used, to avoid problems with cell cycle arrest or premature exit, Fig. 2A, B and the description in the text are misleading. They suggest that the authors generated cells, of which up to 50% completely lack centrioles (5 days). First, the claim that there is no significant difference between 2 and 5 day treatments must be a problem with low n (only ~50 cells, 2 experiments). It naturally takes multiple cell cycles with inhibited centriole duplication to completely deplete existing centrioles from a population of cells (e.g. Wong et al. 2015, Science; Lambrus et al., 2016, J Cell Biol). So longer treatment should result in fewer centrioles per cell. Second, after only 2 days of centrinone treatment, only a minority of neurons seems to have only 1 or 0 centrioles (Fig. S2F), but the exact percentages are still not shown. The authors should include in Fig. 2 quantification of centriole number in neurons derived from NPCs after 2 days in centrinone, as used throughout the study (as shown for SAS6 KO in Fig. S2H).

In this study we used a two days treatment of Centrinone-B not to avoid problems with cell cycle arrest or premature exit, but rather as a consequence of it. We observed that treating neuronal stem cells with Centrinone-B resulted in terminal cell cycle exit and premature neuronal differentiation after three days, thereby limiting the efficiency of centriole loss by Centrinone-B treatment in this system (FIG S2A-E). Please note that our cultures also contain glia cells which likely represent the non-neuronal population at day 5 in FIG S2D,E rather than proliferative neuronal stem cells, as we previously observed a similar non-neuronal population remaining stable over time when characterizing this system (Lindhout et al. 2020, Elife). The quantifications shown at day 2 in FIG2B represent the percentages of centriole loss by Centrinone-B treatment used throughout the study, similar as for SAS6 KO in FIG S2H. We applied textual changes to a re-revised version of the manuscript to better clarify the issues raised here.

Addressing the above points will help the reader to correctly interpret the results and avoid confusion. In my opinion the technical challenges in this study to produce neurons without centrosome (no centrioles) do not allow to completely settle the controversy about the contribution of the centrosomal MTOC to neuronal differentiation. However, the presented findings are still interesting and relevant, and, with appropriate modifications, would be suitable for publication in EMBO J.

We thank the reviewer for appreciating the findings as well as the relevance presented in this study. We have applied textual changes to the re-revised version of the manuscript to address the comments of the reviewer.
Referee #2:

I believe the authors addressed the main and critical points raised by all reviewers. I support this manuscript to be accepted for publication.

We thank the reviewer for the time to review the revised version of the manuscript and we are pleased to note that all points are considered to be addressed.

Referee #3:

The authors have convincingly addressed all my concerns in their revision. I support publication of their study in EMBO Journal.

We thank the reviewer for the efforts to review the revised version of the manuscript and we are pleased to note that all the concerns raised by the reviewer have been addressed.
Dear Casper,

Thanks for sending me the revised manuscript. I have now had a chance to take a look at everything and all looks good.

I am therefore very pleased to accept the manuscript for publication here.

Congratulations on a nice study

with best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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If you have any questions, please do not hesitate to call or email the Editorial Office. Thank you for your contribution to The EMBO Journal.

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A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner;
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way;
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical duplicates;
- if n > 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified;
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the authorship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (e.g. cell line, species name);
- the analysis and method(s) used to carry out the reported observations and measurements;
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner;
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a statement of how many times the experiment shown was independently replicated in the laboratory.

Definitions of statistical methods and measures:

- common tests, such as t test (please specify whether paired vs. unpaired), simple t tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods sections;
- are tests one-sided or two-sided?
- are there adjustments for multiple comparisons?
- definitions of 'center values' as median or average;
- definitions of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

B- Statistics and general methods

1. a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?  
No statistical methods were used for sample size, as there was no prior expectation of effect size.

1. b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.  
N.A.

2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-tested?  
N.A.

3. Were any steps taken to minimize the effects of subjacent bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.  
No randomization was applied.

4. a. For animal studies, include a statement about randomization even if no randomization was used.  
N.A.

4. b. For animal studies, include a statement about blinding even if no blinding was done.  
N.A.

5. For every figure, are statistical tests justified as appropriate?  
Yes.

6. Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.  
Yes, this was done by default examined by plotting boxplots of the datasets. Non-parametric methods were used if data was not normally distributed.
C- Reagents

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

2. Provide a statement only if it could.

D- Animal Models

1. Provide a “Data Availability” section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g., RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for ‘Data Deposition’.

2. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD002021.

E- Human Subjects

1. Identify the committee(s) approving the study protocol.

2. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the MRAA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.

3. For experiments involving big data, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.

4. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under ‘Reporting Guidelines’. Please confirm compliance.

5. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under ‘Reporting Guidelines’. Please confirm you have followed these guidelines.

F- Data Accessibility

1. Provide a “Data Availability” section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g., RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for ‘Data Deposition’.

2. Data deposition in a public repository is mandatory for:
   - b. Macromolecular structures
   - c. Crystallographic data for small molecules
   - d. Functional genomics data
   - e. Proteomics and molecular interactions

3. For publication of patient photos, include a statement confirming that consent to publish was obtained.

4. Report any restrictions on the availability (and/or on the use) of human data or samples.

5. Report the clinical trial registration number (at ClinicalTrial.gov or equivalent), where applicable.

6. If phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under ‘Reporting Guidelines’. Please confirm you have submitted this list.

7. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under ‘Reporting Guidelines’. Please confirm you have followed these guidelines.

8. Is there an estimate of variation within each group of data?

9. Is there an estimate of variation within each group of data?

10. In compliance with the guidelines.

11. Exemplarily, transparency promotion data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD002021.

12. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD002021.

G- Dual use use of research concern

1. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (AHFS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.