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# ASPM and CITK regulate spindle orientation by affecting the dynamics of astral microtubules

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**Review timeline:**

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## Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 05 January 2016

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, the referees acknowledge the potential interest of the findings. However, all referees also point out several technical concerns and have a number of suggestions for how the study should be strengthened, and I think that all of them should be addressed. In particular, referee 1 suggests further experiments to clarify the role of CITK in neural progenitors (point 1) and to address if CITK functions in spindle symmetry (point 2), which have to be addressed in the revision. Referee 2 suggests to quantify the effects on spindle orientation and to address if CITK affects spindle length and referee 3 proposes to analyze if the disruption of the CITK and ASPM interaction impairs spindle orientation, among other concerns.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your

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responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further. You can submit the revision either as a Scientific Report or as a Research Article. For Scientific Reports, the revised manuscript can contain up to 5 main figures and 5 Expanded View figures. If the revision leads to a manuscript with more than 5 main figures it will be published as a Research Article. In this case the Results and Discussion section can stay as it is now. If a Scientific Report is submitted, these sections have to be combined. This will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. In either case, all materials and methods should be included in the main manuscript file.

Supplementary/additional data: The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

Regarding data quantification, can you please specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends? This information is currently incomplete and must be provided in the figure legends.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel. I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

The manuscript entitled "ASPM and CITK regulate orientation affecting the dynamics of astral microtubules" and submitted by Marta Gai et al. describes new interactions between ASPM and CITK at the spindle mole during mitosis, and how such interactions influence the mode of cell division. CITK was first shown to be involved in cytokinesis, consistently to its expression at the midbody during cell division. Here the authors show that that ASPM is necessary for CITK recruitment at the spindle pole to participate in astral microtubules nucleation and stabilization. The characterization of the role of CITK on astral microtubules seem to be well conducted and convincing. However, I consider that several aspects of the manuscript listed below have to be improved or clarified.

1. The authors have largely focused the introduction and the discussion of the manuscript on the important impact of the imbalance between symmetric and asymmetric cell divisions on the maintenance of the progenitor pool size and the final brain size. Such imbalance has indeed been largely involved in genetic primary microcephaly (MCPH). However, most of the experiments have been conducted in HeLa cells and the transposition of these CITK functional studies to neural progenitors is globally weakly supported. Experiments carried in drosophila neuroblasts are not really informative, since the authors do not address whether asymmetric cell division triggered by

dck depletion changes the fate of the neuroblasts. In this regard, the manuscript would greatly benefit from clarifiying the following issues:
. Is the higher incidence of cell cycle exit described in the mouse CITK mutant embryos associated with premature neurogenesis and/or an increase in Tbr2 positive intermediate progenitors?
. The presence of BrdU+/Ki67- in the VZ 24 hours after the BrdU injection is striking and needs to be explained. Do these cells correspond to neurons unable to migrate and leave the VZ?
. page12, the discussion related to the relative impact of impaired cytokinesis and oblique division axis is rather confusing. First, the authors mention " .... abnormal cytokinesis and apoptosis could contribute to ASPM mediated microcephaly. However, a significant increase of the latter events has not been documented (no reference cited) in vivo neither in humans nor in mouse ». Then comes a sentence suggesting an apposite point of view : « On this basis, it is therefore possible that the microcephaly produced in mammals by CITK loss is not only due to cytokinesis failure and apoptosis, but also to a reduced expansion of the cortical neural stem cells pool ». To state on the relative impact of impaired cytokinesis and oblique divsion, the authors have either to refer to published data, or to assess impaired citokinesis and cell death in CITK mutant embryos.

2. The authors do not refer to data reported in Delaunay et al., Cell Reports, 2014, which show that mitotic spindle-size asymmetry, negatively controlled by the Wnt pathway, is associated with asymmetric neurogenic division. ASPM has been shown to act upstream of the Wnt pathway to trigger symmetric divisions of cortical progenitors, and could thus be also involved in the control of the spindle symmetry. With this regard, in addition to the angle of division axis, the authors should assess whether CITK loss of function impacts spindle symmetry or not. In particular, they should address whether the loss of microtubules observed in the context of CITK knock-down is equivalent in both spindle sides.

Minor points
3. Does ASPM and CITK simultaneous knock-down have a cumulative effect on cell division?

4. According to Fig. 2F, the PLA staining performed in cells expressing ASPM-GFP generates signal in the cytoplasm and not only at the spindle pole. Is there any explaination for such a staining? Further, although the authors show Fig. 2D clear double immunostaining of the endogenous ASPM and CITK proteins, but they provide PLA staining with GFP-ASPM and endogenous CITK. Is there any raison, technical or else, for not providing demonstration of interactions between both endogenous proteins?

5. Page 6, second line, and Figure 1 legend: the discrepancy on the time of the BrdU pulse, mentioned respectively at E14.5 and E13.5, has to be corrected.

5. The beginning of the discussion provides a too long description of the literature related to mechanisms controlling symmetric versus asymmetric cell division in neural progenitors.

Referee #2:

In this manuscript Gai and colleagues show that ASPM and citron kinase regulate spindle orientation via the regulation of astral microtubule dyanmics. ASPM is encoded by the gene most frequently mutated in microcephaly, and is known to contribute to control of spindle orientation and spindle focusing during mitosis. Here the authors study the role of citron kinase in this process. This kinase had been previously implicated in the control of midbody formation during cytokinesis, and it is a known interactor of ASPM. The authors show that citron kinase weakly binds to spindle poles during early stages of mitosis in an ASPM-dependent manner and that depletion of citron kinase leads to spindle orientation defects in human tissue culture cells, in an neuronal progenitors in mice brain, and in drosophila neuroblasts. The authors then further show that the overexpression of citron kinase can rescue the spindle orientation phenotype seen after the depletion of ASPM, linking the two protein in the control of this process. Finally the author report that the depletion of either protein reduces the stability of astral microtubules, and that the addition of the microtubule-stabilizer taxol rescues the spindle orientation phenotype.

The manuscript is interesting as is links for the first time citron kinase to the control of spindle orientation. Moreover the authors have good data indicating that citron kinase acts downstream of

ASPM. This therefore expands our knowledge on this essential process, which is hypothesized to be linked to the prevention of microcephaly in human patients. The data are in general of good technical quality, nevertheless the manuscript has some punctual weaknesses that should be addressed in order to strengthen this study.

Major points:
The data presented in Figure 1F-H are a bit arbitrary, since it is not clear how the authors when a division is oblique, and when it is parallel to the growth surface. A quantification of the angle, as shown in Figure 1I and J, would be more informative. A second issue in Figure 1, is that the spindle orientation defect is rather mild, when compared to other treatments leading to spindle orientation defects (see for example Toyoshima and Nishida, EMBO J, 2007), as then angle only increases from 8 to 12 degrees. One important control would be to measure how the depletion of citron kinase affects spindle length, since a reduction in spindle length at a constant difference in Z will lead to a small increase in the spindle angle, even if spindle orientation is not affected. The authors should moreover discuss the fact that the observed phenotype is rather mild.

In Figure 2, the authors report that citron kinase localizes to spindle poles and that it requires ASPM to do so. Instead of just reporting whether citron kinase is present at spindle poles or not in ASPM-depleted cells, it would be more informative to quantify the relative levels of citron kinase at spindle pole when compared to control-depleted cells.

In Figure 3, the authors report that depletion of ASPM or citron kinase leads to a reduction in the number and the length of astral microtubules. A first issue is that some measurements (Fig3C) are only supported by two independent experiments, yet the authors report p-values of less than 0.001 for two-tailed t-test. Such a low p-value seems implausible, given that the measurements are only based on 2 independent experiments. I am therefore wondering whether the authors used n=2 for their statistical evaluation and calculations of error bars or did they use n=50, the number of cells? The authors should make sure to use for all statistical tests the number of independent experiment. Moreover, in case they have only two data points, they should not use bar graphs, but rather report the individual values in a scatter plot, as this would be more informative. Furthermore, a third independent experiment might also be helpful. A second problem, is that in Figure 3E and in the other figures, the authors always reported the absolute spindle angle, yet in Figure 3D they measure the "fold increase in mitotic angle". To allow a comparison of all results, the authors should always show the absolute spindle angle. Finally, there is a discrepancy between the pictures shown in Figure 3A, in which one can see a clear reduction in the number of astral microtubules, and the pictures shown in Figure S2A (p150 staining and dynein staining), in which the astral microtubules look very much alike in control-depleted cells and cells depleted of citron kinase. How do the authors reconcile this discrepancy?

Finally, in Figure 4 the authors aim to identify the specific microtubule parameter that is changed in cells lacking ASPM or citron kinase. The authors report a change in microtubule nucleation. The data presented in Figure 4D and E does not support this data very well. First the authors should show the cells in Figure 4D with a much larger magnification, one can barely see the asters. Second, how do the authors explain that depletion of citron kinase does not affect microtubule nucleation after 5mins, yet has an effect after 10mins, given that microtubule nucleation is usually a very rapid process? If there was a difference one would expect a significant difference at the earliest time point, not at later time points. The authors might therefore want to look at even earlier time points (1 or 2 mins), to see if microtubule nucleation at the centrosomes is significantly affected in cells lacking citron kinase. This would also be cleaner, as in later time points microtubules can also be nucleated from other structures, such as chromosomes or kinetochores, which makes the quantification much more difficult.

Referee #3:

In this manuscript, Gai et al. reported a new mechanism involving ASPM and CITK that regulates spindle orientation. Built on the previous evidence of a physical interaction between ASPM and CITK, the authors found that, like ASPM, CITK is a conserved regulator of spindle orientation based on mouse and fly mutant analysis. As expected, a pool of CITK is localized at the spindle pole in an ASPM-dependent manner. Moreover, CITK acts downstream of ASPM to control spindle

orientation by regulating dynamic astral microtubule (MT) organization.

Main comments:
1. As stated by the authors, spindle orientation control was traditionally believed to be one of the key factors in cortical neurogenesis. However, recent evidence has raised critical issues with this theory. Recent analysis of LGN mutant and Sas4p53 double mutant brains suggest that the spindle orientation may not be essential for cortical neurogenesis (Konno et al., Nat. Cell Biol. 2008; Insolera et al., Nat. Neurosci. 2014; Homem et al., Nat. Rev. Neurosci. 2015). In addition, a recent study on ASPM mutant brain suggests a cell cycle centric mechanism underlying microcephaly, independent of mitotic spindle orientation (Capecchi and Pozner, Nat. Commun. 2015). The authors should take into consideration the new evidence.

2. Figure 1C: Without knowing the overall cortical distribution of BrdU+ cells, it is difficult to distinguish an increase in cell cycle exit from neuronal migration defects based on the relative increase of BrdU+/Ki67- cells in the VZ and IZ. In addition, the authors should provide representative images of BrdU/Ki67 staining.

3. Figure 2F: The panel showing CITK staining, described in the caption, is absent. Moreover, in the proximity ligation assay (PLA), the majority of positive spots indicating a close proximity between ASPM and CITK spread throughout the cells, and only appear on one spindle pole but not the other. It calls into the question the validity of PLA results.

4. Figure 4C and D: Given that the spindle-pole localization of CITR depends on ASPM, it is difficult to comprehend that simply overexpression of CITR can rescue ASPM knockdown phenotypes in the astral MT organization. Alternatively, it should be important to determine whether a disruption of the interaction between ASPM and CITR impairs the spindle orientation. Another possible way to rescue the ASPM knockdown phenotype is to modestly express CITR fused with a spindle-pole targeting motif.

Minor comments:
1. For all siRNA experiments, the rescue experiments with the siRNA-resistant form of genes should be included to confirm the specificity of siRNA.

2. There are many typos in the manuscript and the authors should revise the text carefully.

1st Revision - authors' response 04 June 2016

Thanks for giving us the opportunity to revise our manuscript, n. EMBOR-2015-41823, entitled: “ASPM and CITK regulate spindle orientation by affecting the dynamics of astral microtubules” and for extending the deadline for re-submission.

We would also like to thank the Referees for their constructive comments, which helped us to improve the manuscript.

We performed all the experiments required to addressed their concerns. The results were all informative, with the only exception of those undertaken to disrupt the interaction between CITK and ASPM. Indeed, it turned out that the association of this two proteins may involve multiple interaction domains. Therefore, we did not find a single sequence that could be expressed in cells to disrupt their association. If required, we can provide the results of these experiments, but we would not include them in the manuscript because they would not offer a significant contribution. All the new informative evidence has been included in the revised manuscript, which we shortened and simplified to fit the Report format.

We hope that this improved manuscript may be deemed suitable for publication in EMBO Reports.

**Point by point response to Referees' comments**

**Referee #1:**

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We thank the Referee for the overall positive evaluation of the manuscript.

Comment:

1. The authors have largely focused the introduction and the discussion of the manuscript on the important impact of the imbalance between symmetric and asymmetric cell div isions on the maintenance of the progenitor pool size and the final brain size. Such imbalance has indeed been largely involved in genetic primary microcephaly (MCPH). However, most of the experiments have been conducted in HeLa cells and the transposition of these CITK functional studies to neural progenitors is globally weakly supported. Experiments carried in drosophila neuroblasts are not really informative, since the authors do not address whether asymmetric cell div ision triggered by dck depletion changes the fate of the neuroblasts. In this regard, the manuscript would greatly benefit from clarifiying the following issues:

Is the higher incidence of cell cycle exit described in the mouse CITK mutant embryos associated with premature neurogenesis and/or an increase in Tbr2 positive intermediate progenitors?

Response:

To address these issues, we stained brain sections of E14.5 embryos, injected with BrdU at E13.5, for DNA, BrdU, Ki67 and either anti-Tbr2 or anti Tubb3 (TuJ) antibodies. The results are show in the new Fig. 1. The density of neurons in the SVZ/VZ of CitK -/- mice is not increased, but rather is reduced to half of the control. Considering this reduction, we could not obtain reasonable statistics of newly generated neurons in the mutants, and therefore we show in figure 1E only neurons' total number. Although this result would seem sufficient to deduce that the number of neurons is not increased, it must be taken with great caution. We previously demonstrated (Sgrò et. al, 2016) that in CitK -/- mice most of the cells that undergo apoptosis are in fact neurons. For this reason we think that it is not possible to reasonably assess whether neurogenesis is increased. On the other hand, our analysis revealed a significant increase of BrdU+/Tbr2+ cells (Fig. 1F), which is consistent with an increased production of basal progenitors from apical progenitors' division.

Comment:

The presence of BrdU+/Ki67- in the VZ 24 hours after the BrdU injection is striking and needs to be explained. Do these cells correspond to neurons unable to migrate and leave the VZ?

Response:

The BrdU+/Ki67- cells detected in CitK -/- mice are a mixed population. Only few of them are neurons (<10%), a result consistent with the total reduction of neuron density in these areas (see above). Some of these cells are Tbr2-positive, some are Pax6 positve (data not shown) and some are negative for all tested markers, more or less with similar frequency. These results suggest that, in addition to apoptosis and increased generation of basal progenitors, CitK neural progenitors may be characterized by pre-mature exit from cell cycle. The latter result further complicates the interpretation of cell fate disturbance in CitK -/- mice.

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Comment:

. page12, the discussion related to the relative impact of impaired cytokinesis and oblique div ision axis is rather confusing. First, the authors mention " .... abnormal cytokinesis and apoptosis could contribute to ASPM mediated microcephaly. However, a significant increase of the latter events has not been documented (no reference cited) in v ivo neither in humans nor in mouse ». Then comes a sentence suggesting an apposite point of v iew : « On this basis, it is therefore possible that the microcephaly produced in mammals by CITK loss is not only due to cytokinesis failure and apoptosis, but also to a reduced expansion of the cortical neural stem cells pool ». To state on the relative impact of impaired cytokinesis and oblique divsion, the authors have either to refer to published data, or to assess impaired citokinesis and cell death in CITK mutant embryos.

Response:

This problem was probably caused by the fact that, as underscored by Referee 3, in the previous version of the manuscript we over-emphasized the importance of spindle orientation for cell fate choice. In consideration of the recent reports suggesting that in mammals the relationship between spindle orientation and cell fate may only be correlative (see major points of Referee 3), and of the complex cell fate phenotype which we were faced with(see above), we have strongly attenuated all the statements linking spindle orientation with cell fate choice.
We would like to underscore that the main focus of this report was to analyze the relationship between CITK and ASPM in spindle orientation. To this regard, the important message which we would like to convey with Fig. 1 is that spindle orientation is disturbed in vivo, in both mammals and insects. How CITK and ASPM may cooperate to influence cell fate remains an open issue. We hope that the new, simplified text does not incur anymore in the previous ambiguity.

Comment:

2. The authors do not refer to data reported in Delaunay et al., Cell Reports, 2014, which show that mitotic spindle-size asymmetry, negatively controlled by the Wnt pathway, is associated with asymmetric neurogenic div ision. ASPM has been shown to act upstream of the Wnt pathway to trigger symmetric div isions of cortical progenitors, and could thus be also involved in the control of the spindle symmetry. With this regard, in addition to the angle of div ision axis, the authors should assess whether CITK loss of function impacts spindle symmetry or not. In particular, they should address whether the loss of microtubules observed in the context of CITK knock-down is equivalent in both spindle sides.

Response:

We addressed this issue in HeLa cells and show the results in the new figure EV3. Under our experimental conditions, in spite of clear spindle positioning phenotypes, we did not detect significant differences in spindle asymmetry between control cells and cells depleted of CITK or ASPM.

Minor points

Comment:

3. Does ASPM and CITK simultaneous knock-down have a cumulative effect on cell division?

Response:

ASPM and CITK simultaneous knock-down doesn't have a cumulative effect on spindle orientation. The result is shown in Fig. 2F and supports the hypothesis that CITK and ASPM are in the same pathway.

Comment:

4. According to Fig. 2F, the PLA staining performed in cells expressing ASPM-GFP generates signal in the cytoplasm and not only at the spindle pole. Is there any explaination for such a

staining? Further, although the authors show Fig. 2D clear double immunostaining of the endogenous ASPM and CITK proteins, but they provide PLA staining with GFP-ASPM and endogenous CITK. Is there any raison, technical or else, for not providing demonstration of interactions between both endogenous proteins?

Response:

PLA is a technique characterized by single molecule sensitivity, due to strong amplification. We think that the detected signal is specific, because the negative control, which only differs from positive sample for the absence of the GFP tag connected to ASPM, is completely negative. In our view, this result indicates that CITK and ASPM interact not only at the spindle but also in the cytoplasm. This interpretation is consistent with the fact that, before the beginning of anaphase, a large pool of CITK and a relatively small pool of ASPM are cytoplasmic. Consider that no pre-extraction is performed with this technique.

Comment:

5. Page 6, second line, and Figure 1 legend: the discrepancy on the time of the BrdU pulse, mentioned respectively at E14.5 and E13.5, has to be corrected.

Response:

Corrected

Comment:

5. The beginning of the discussion prov ides a too long description of the literature related to mechanisms controlling symmetric versus asymmetric cell division in neural progenitors.

Response:

The discussion has been combined with results to reduce redundancy

**Referee #2:**

In this manuscript Gai and colleagues show that ASPM and citron kinase regulate spindle orientation via the regulation of astral microtubule dyanmics. ASPM is encoded by the gene most frequently mutated in microcephaly, and is known to contribute to control of spindle orientation and spindle focusing during mitosis. Here the authors study the role of citron kinase in this process. This kinase had been previously implicated in the control of midbody formation during cytokinesis, and it is a known interactor of ASPM. The authors show that citron kinase weakly binds to spindle poles during early stages of mitosis in an ASPM-dependent manner and that depletion of citron kinase leads to spindle orientation defects in human tissue culture cells, in an neuronal progenitors in mice brain, and in drosophila neuroblasts. The authors then further show that the overexpression of citron kinase can rescue the spindle orientation phenotype seen after the depletion of ASPM, linking the two protein in the control of this process. Finally the author report that the depletion of either protein reduces the stability of astral microtubules, and that the addition of the microtubule-stabilizer taxol rescues the spindle orientation phenotype.
The manuscript is interesting as is links for the first time citron kinase to the control of spindle orientation. Moreover the authors have good data indicating that citron kinase acts downstream of ASPM. This therefore expands our knowledge on this essential process, which is hypothesized to be linked to the prevention of microcephaly in human patients. The data are in general of good technical quality, nevertheless the manuscript has some punctual weaknesses that should be addressed in order to strengthen this study.

We thank the Referee for the overall positive evaluation of the manuscript.

Major points:

Comment:

The data presented in Figure 1F-H are a bit arbitrary, since it is not clear how the authors when a div ision is oblique, and when it is parallel to the growth surface. A quantification of the angle, as shown in Figure 1I and J, would be more informative.

Response:

We consider oblique a division showing uneven timing of daughter cell flattening onto the substrate after mitosis and with one daughter cell dividing outside the plane. This phenotype is similar to that of U2OS treated with ASPM siRNA (Higgins J. et al. 2010) and it has been described also for other proteins involved in mitotic spindle orientation (Gallini S. et al. 2016; Delaval B. 2011). A quantification of angles on wide field time lapse movies is not possible. We provide this quantification for cells analyzed by confocal microscopy in fixed samples. In the revised version of the manuscript all these panels are reported in figure 2. We tried to better describe the phenotype in the results and in the figure legends.

Comment:

A second issue in Figure 1, is that the spindle orientation defect is rather mild, when compared to other treatments leading to spindle orientation defects (see for example Toyoshima and Nishida, EMBO J, 2007), as then angle only increases from 8 to 12 degrees. One important control would be to measure how the depletion of citron kinase affects spindle length, since a reduction in spindle length at a constant difference in Z will lead to a small increase in the spindle angle, even if spindle orientation is not affected. The authors should moreover discuss the fact that the observed phenotype is rather mild.

Response:

We quantified spindle length and it is not affected by CITK depletion (Figure EV3A). The misorientation phenotype which we observed is less severe than the phenotype resulting from β1-integrin loss (Toyoshima F et al. EMBO J. 2007), but is comparable with the one reported for depletion of the microcephaly protein WDR62 (Bogoyevitch MA et al. J Cell Sci. 2012) and for partial Aurora-A inactivation (Gallini et al. Curr Biol. 2016). Since the phenotype is quantified, with statistically significant differences, and since we did not say that it is severe, we would prefer not to change the text on this point and leave the reader conclude about severity.

Comment:

In Figure 2, the authors report that citron kinase localizes to spindle poles and that it requires ASPM to do so. Instead of just reporting whether citron kinase is present at spindle poles or not in ASPM-depleted cells, it would be more informative to quantify the relative levels of citron kinase at spindle pole when compared to control-depleted cells.

Response:

As suggested by the Referee, we quantified the ratio of CITK at spindle poles versus total cell mean intensity, in control and ASPM-depleted cells (Fig. 3I). Even with this parameter, we observed a statistically significant reduction.

Comment:

In Figure 3, the authors report that depletion of ASPM or citron kinase leads to a reduction in the number and the length of astral microtubules. A first issue is that some measurements (Fig3C) are only supported by two independent experiments, yet the authors report p-values of less than 0.001 for two-tailed t-test. Such a low pvalue seems implausible, given that the measurements are only based on 2 independent experiments. I am therefore wondering whether the authors used n=2 for their statistical evaluation and calculations of error bars or did they use n=50, the number of cells? The authors should make sure to use for all statistical tests the number of independent experiment. Moreover, in case they have only two data points, they should not use bar graphs, but rather report

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the individual values in a scatter plot, as this would be more informative. Furthermore, a third independent experiment might also be helpful.

Response:

We agree with the Referee that this point could generate confusion. Histograms and error bars were obtained by averaging not two data points, but all the cells counted in the two replicas (a comparable number of cells was counted in every replica). We now state clearly this point in the last section of Materials and Methods (Statistics). In addition, to avoid any misunderstanding, we performed a new replicate for all the experiments that were based on only two replicas.

Comment:

A second problem, is that in Figure 3E and in the other figures, the authors always reported the absolute spindle angle, yet in Figure 3D they measure the "fold increase in mitotic angle". To allow a comparison of all results, the authors should always show the absolute spindle angle.

Response:

As suggested by the Referee, we now show in panel D of the new figure (Fig. 4) the absolute spindle angle.

Comment:

Finally, there is a discrepancy between the pictures shown in Figure 3A, in which one can see a clear reduction in the number of astral microtubules, and the pictures shown in Figure S2A (p150 staining and dynein staining), in which the astral microtubules look very much alike in control-depleted cells and cells depleted of citron kinase. How do the authors reconcile this discrepancy?

Response:

The problem was caused by image choice. In the previous version, we wanted to show control and CITK-depleted cells with mitotic spindle perfectly parallel to the surface, to avoid the impact of the visual differences deriving from a tilted spindle. These cells are easy to find in the control population, much more difficult to find in the CITK-depleted population. At the end, we found some examples, which we showed.
However, it turns out that cells with horizontal spindle are not representative of the population, because they show a number of astral microtubules very similar to control cells, and different from cells with tilted spindle, which are the largest population. We now show two more representative examples, in which the spindle is almost planar but astral microtubules are reduced (Fig. EV2A).

Comment:

Finally, in Figure 4 the authors aim to identify the specific microtubule parameter that is changed in cells lacking ASPM or citron kinase. The authors report a change in microtubule nucleation. The data presented in Figure 4D and E does not support this data very well. First the authors should show the cells in Figure 4D with a much larger magnification, one can barely see the asters.

Response:

The Referee is right, the suggested change has been made (Fig. 5D)

Comment:

Second, how do the authors explain that depletion of citron kinase does not affect microtubule nucleation after 5mins, yet has an effect after 10mins, given that microtubule nucleation is usually a very rapid process? If there was a difference one would expect a significant difference at the earliest time point, not at later time points. The authors might therefore want to look at even earlier time points (1 or 2 mins), to see if microtubule nucleation at the centrosomes is significantly affected in cells lacking citron kinase. This would also be cleaner, as in later time points microtubules can also

be nucleated from other structures, such as chromosomes or kinetochores, which makes the quantification much more difficult.

Response:

Also on this point we agree with the Referee. Indeed, most studies that use this assay analyze microtubule nucleation in the first few minutes. We realized that the reason of our discrepant kinetic was a technical problem in the protocol, resulting in slow rise of the temperature. We therefore optimized experimental setting in order to have a more rapid shift of temperature from 4°C to 37°C and, as suggested, analyzed microtubule nucleation 1 and 2 minutes after release. We obtained cleaner results (Fig. 5E) with a delay in MT regrowth after 1 minute in cells lacking citron kinase. 2 minutes after release almost all cells have nucleated new MT from centrosomes, but aster size in significantly reduced in CITK depleted versus control cells. We thank the Referee for having identified this problem.

**Referee #3:**

Comment:
In this manuscript, Gai et al. reported a new mechanism involving ASPM and CITK that regulates spindle orientation. Built on the previous evidence of a physical interaction between ASPM and CITK, the authors found that, like ASPM, CITK is a conserved regulator of spindle orientation based on mouse and fly mutant analysis. As expected, a pool of CITK is localized at the spindle pole in an ASPM-dependent manner. Moreover, CITK acts downstream of ASPM to control spindle orientation by regulating dynamic astral
microtubule (MT) organization.

Main comments:

1. As stated by the authors, spindle orientation control was traditionally believed to be one of the key factors in cortical neurogenesis. However, recent evidence has raised critical issues with this theory. Recent analysis of LGN mutant and Sas4p53 double mutant brains suggest that the spindle orientation may not be essential for cortical neurogenesis (Konno et al., Nat. Cell Biol. 2008; Insolera et al., Nat. Neurosci. 2014; Homem et al., Nat. Rev. Neurosci. 2015). In addition, a recent study on ASPM mutant brain suggests a cell cycle centric mechanism underlying microcephaly, independent of mitotic spindle orientation (Capecchi and Pozner, Nat. Commun. 2015). The authors should take into consideration the new ev idence.

Response:

We agree with the Referee that in the previous version of the manuscript we over-emphasized the importance of spindle orientation for cell fate choice. Moreover, the fact that we did not consider in full the work cited by the Referee was an important miss on our side. The idea behind our formulation was to provide the reader with the main reasons that make interesting to look at spindle orientation. However, this inevitably conveyed the idea that our work was biased towards a unilateral interpretation of spindle orientation relevance for cell fate choice. Including in the introduction and discussion the evidence that argues against relevance of spindle orientation in cell fate determination is therefore essential.
In this revised version, also considering the complex cell fate phenotype which we were faced with, we have strongly attenuated all the statements linking spindle orientation with cell fate choice. We are very grateful to the Referee for pointing out these problems.
That said, we would also like to underscore that the main focus of this report was to analyze the relationship between CITK and ASPM in spindle orientation, as the Referee correctly highlighted.

Comment:

2. Figure 1C: Without knowing the overall cortical distribution of BrdU+ cells, it is difficult to distinguish an increase in cell cycle exit from neuronal migration defects based on the relative increase of BrdU+/Ki67- cells in the VZ and IZ. In addition, the authors should prov ide representative images of BrdU/Ki67 staining.

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Response:
We now show full sections of E14.5 embryos, injected with BrdU at E13.5, stained for DNA, BrdU, Ki67 and either anti-Tbr2. We also stained sections with anti Tubb3 (TuJ) antibodies The density of neurons in the SVZ/VZ of CitK -/- mice is not increased, but rather is reduced to half of the control, arguing against a neuronal migration defect. Although this result would seem sufficient to deduce that the number of neurons is not increased, it must be taken with great caution. Indeed, in CitK -/- mice most of the cells that undergo apoptosis are in fact neurons (Sgrò et. Al, 2016). For this reason, we think that it is not possible to assess whether neurogenesis is altered. On the other hand, our analysis revealed a significant increase of BrdU+/Tbr2+ cells (Fig. 1F), which is consistent with an increased production of basal progenitors. The BrdU+/Ki67- cells detected in CitK -/- mice are a mixed population. Only few of them are neurons (<10%), a result consistent with the total reduction of neuron density. Some of these cells are Tbr2-positive, some are Pax6 positive (data not shown) and some are negative for all tested markers. These results suggest that, in addition to apoptosis and increased generation of basal progenitors, CitK neural progenitors may be characterized by premature exit of progenitors from cell cycle, further complicating the interpretation of cell fate disturbance in CitK -/- mice.

Comment:

3. Figure 2F: The panel showing CITK staining, described in the caption, is absent.

Response:

The Referee is right. What created confusion was the term 'immunostained'. Indeed, in PLA cells are only incubated with primary reagents, while the interaction is revealed with specific secondary reagents linked to DNA oligonucleotides, which are then amplified by rolling-cyrcle DNA replication. The text has been amended.

Comment:

Moreover, in the proximity ligation assay (PLA), the majority of positive spots indicating a close proximity between ASPM and CITK spread throughout the cells, and only appear on one spindle pole but not the other. It calls into the question the validity of PLA results.

Response:

PLA is a technique characterized by single molecule sensitivity, due to strong amplification. We think that the detected signal is specific, because the negative control, which only differs from positive sample for the absence of the GFP tag connected to ASPM, is completely negative. In our view, this result indicates that CITK and ASPM interact not only at the spindle but also in the cytoplasm. This interpretation is consistent with the fact that, before the beginning of anaphase, a large pool of CITK and a relatively small pool of ASPM are cytoplasmic. Consider that no pre-extraction is performed with this technique.

Comment:

4. Figure 4C and D: Given that the spindle-pole localization of CITR depends on ASPM, it is difficult to comprehend that simply overexpression of CITR can rescue ASPM knockdown phenotypes in the astral MT organization. Alternatively, it should be important to determine whether a disruption of the interaction between ASPM and CITR impairs the spindle orientation. Another possible way to rescue the ASPM knockdown phenotype is to modestly express CITR fused with a spindle-pole targeting motif.

Response:

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We have tried very hard to identify sequences that may disrupt the ASPM/CITK interaction. However, we had to face the problem that these two proteins seem to interact through multiple regions, and therefore it was not possible to express one sequence capable of disrupting the interaction. Moreover, in the time frame of this revision, we were not able to setup the second experiment suggested by the Referee, which is technically demanding,
Nevertheless, we think that the rescue of ASPM phenotype by CITK could be explained by recent findings showing that CITK has the potential to bind microtubules directly or through other proteins (Bassi et al., 2013; Bassi et al., 2011). Therefore, when CITK is expressed at physiological levels, the presence of ASPM could be the limiting factor for recruiting it to the spindle and for its activity on spindle orientation. Under overexpression conditions, the avidity of microtubules and microtubule-associated proteins for CITK could compensate the loss of affinity produced by ASPM depletion. This possibility is suggested in the new text.

Minor comments:

Comment:

1. For all siRNA experiments, the rescue experiments with the siRNA-resistant form of genes should be included to confirm the specificity of siRNA.

Response:

We have validated the specificity of the RNAi phenotype by using two different sequences and by performing a rescue experiment (Fig. EV1). In addition, we observed the same phenotypes in vivo, in two phylogenetically distant species (Fig 1). Repeating all the experiments in parallel with a rescue plasmid would have been too demanding for our laboratory.

Comment:

2. There are many typos in the manuscript and the authors should revise the text carefully.

Response:

We have made our best to simplify language and correct typos.

2nd Editorial Decision 28 June 2016

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Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, referee 1 and 2 now support publication while referee 3 still raises important concerns and does not recommend publication of the manuscript in its current form. He/she is skeptical about the conclusions drawn concerning neuronal migration defects and considers experiments probing the significance of the ASPM-CITK interaction crucial.

Given the contrasting referee reports we have decided to give you the exceptional possibility of another round of revision to address the concerns regarding the neuronal migration defects (point 2).

Upon further discussion with the referees we think that the interaction between ASPM and CITK and the failure to disrupt its interaction can and should be further discussed in the text and the existence of multiple interaction domains mentioned (point 4). Alternative pathways to localize CITK to the spindle apart from ASPM that explain the rescue in absence of ASPM should be further discussed.

I think that the quantification of the percentage of neurons in SVZ/VZ relative to the total neuron population can be done using existing stainings and sections and hence the revision should not take more than 2 weeks. Yet, I want to highlight the fact that we have a 6 months scooping protection. In your case a first decision had been made on the manuscript on the 5th January 2016, which means that by 5th July 2016 this manuscript would have to be accepted. While I think that the referee's

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concern can be addressed, I can also see that the study will not be in an acceptable form before this time. Given this policy, I will therefore have to check the novelty of the manuscript again at the time of the submission of your revision, but I do not foresee any issues as at the moment I cannot find a new compromising study on this topic.

Please let me know if you have any further questions or need more time for the revision. I am looking forward to receiving a revised version of your manuscript.

REFEREE REPORTS

Referee #1:

All my comments have been adequately taken into comments by the Authors

Referee #2:

The authors have done a good job at addressing the reviewers comment, and the manuscript is now suitable for publication.

Referee #3:

I have the remaining concerns regarding the authors' revision:

Previous Main Comments Point 2:

a. The authors found that the density of neurons is reduced in SVZ/VZ of CITK -/- mice, and argue that this evidence would exclude the possibility of neuronal migration defects.

The authors should instead quantify the percentage of neurons in SVZ/VZ relative to the total neuron population in order to rule out any neuronal migration defects. In addition, given the noticeable apoptosis and significant reduction of TBR2+ cells in CITK -/- mice (Figure 1C and 1E; contradictory to the authors' conclusion of an increase of BrdU+/ TBR2+ cells), there may be a reduction in the overall cell population. Therefore, a reduced density of neurons in VZ/SVZ may not be sufficient to determine if fewer neurons are produced and/or neurons cannot migrate properly.

b. The authors cited their 2016 paper (Sgro et al., 2016) and claimed that the majority of cell death occurs in neurons, therefore it is not possible to assess whether neurogenesis is altered.

Based on the 2016 paper, ~60% TUNEL+ cells are negative for any markers (Pax6, Tbr2 or Tuj1), and only 35% TUNEL+ cells are Tuj1+.

c. The authors claimed that there is a significant increase of TBR2+ BrdU+ cell in total BrdU+ cells, suggesting an increased production of TBR2+ cells.

The overall population of TBR2+ cells was obviously reduced in Figure 1C, inconsistent with an increased production of TBR2+ cells. The increase of TBR2+ BrdU+ cells may be alternatively explained as a result of even greater reduction of total BrdU+ neurons or a delay in cell progression of TBR2+ cells

d. They found BrdU+ Ki67- cells in CITK -/- mice are a mixed population, with only <10% being neurons, and some are PAX6+ and TBR2+.

This is confusing, because PAX6+ and TBR2+ should rarely be Ki67-, and all Ki67- cells should be neurons.

Previous Main Comments Point 4:

The authors could not do the experiment of disrupting ASPM-CITK interaction to assess spindle misorientation, as they claim that these two proteins interact through multiple regions and could not define a single concrete sequence to disrupt the interaction. For the other experiment of targeting CITK with centrosome localization sequence to rescue ASPM phenotype, they cite time restraint for not doing the experiment.

This is one of the most essential experiments to demonstrate the significance of the functional link between ASPM and CITK in spindle orientation. Without this data, the strength of this paper is greatly compromised.

2nd Revision - authors' response 08 July 2016

First of all, I would like to thank you for the exceptional opportunity which you granted us to submit a second revision of our manuscript. As requested we quantified the percentage of neurons in SVZ/VZ relative to the total neuron population in the wild type and in the KO and we found no significant difference, further supporting the conclusion that the absence of CITK does not produce major problems in neuronal migration. Concerning the second point, we have decided to include the data of the immunoprecipitation experiments, showing that both the amino- and the carboxi-terminal halves of CITK are capable of interacting with the C-terminal region of ASPM. The results are shown in a new supplementary figure (EV2). Finally, we have discussed more in depth the possible pathways that could justify the phenotypic rescue of ASPM depletion by CITK overexpression. Also, we have addressed the editorial points. Included, please find a point by point response to the issues raised by Referee 3 on the previous revision.

**Response to comments of Referee 3 on revised manuscript**

**Comment:**
Previous Main Comments Point 2:
a. The authors found that the density of neurons is reduced in SVZ/VZ of CITK -/- mice, and argue that this evidence would exclude the possibility of neuronal migration defects. The authors should instead quantify the percentage of neurons in SVZ/VZ relative to the total neuron population in order to rule out any neuronal migration defects. In addition, given the noticeable apoptosis and significant reduction of TBR2+ cells in CITK -/- mice (Figure 1C and 1E; contradictory to the authors' conclusion of an increase of BrdU+/ TBR2+ cells), there may be a reduction in the overall cell population. Therefore, a reduced density of neurons in VZ/SVZ may not be sufficient to determine if fewer neurons are produced and/or neurons cannot migrate properly.

**Response:**
We have performed the requested quantification. No significant differences were detected between control and CITK-knockout samples (Fig 1E), supporting the conclusion that the absence of CITK does not produce major problems in neuronal migration.

**Comment:**
b. The authors cited their 2016 paper (Sgro et al., 2016) and claimed that the majority of cell death occurs in neurons, therefore it is not possible to assess whether neurogenesis is altered.

Based on the 2016 paper, ~60% TUNEL+ cells are negative for any markers (Pax6, Tbr2 or Tuj1), and only 35% TUNEL+ cells are Tuj1+.

**Response:**
The Referee is right, but we think that the quantification of differentiation markers in apoptotic cells must be taken with great caution, because of the massive proteolytic processes occurring in apoptotic cells. Marker negativity in these cells may just be a consequence of proteolytic disappearance. With this caveat, the large majority of cells in which we could detect the mentioned cell-identity markers are TuJ1+.

**Comment:**
c. The authors claimed that there is a significant increase of TBR2+ BrdU+ cell in total BrdU+ cells, suggesting an increased production of TBR2+ cells.

The overall population of TBR2+ cells was obviously reduced in Figure 1C, inconsistent with an increased production of TBR2+ cells. The increase of TBR2+ BrdU+ cells may be alternatively explained as a result of even greater reduction of total BrdU+ neurons or a delay in cell progression of TBR2+ cells

**Response**
We have quantified the overall population of Tbr2+ cells and there is no difference between control and knockout mice (result included in the new text, page 6). The impression of the Referee was probably due to the fact that Tbr2+ cells are more packed in the knockout than in the control.

**Comment:**
d. They found BrdU+ Ki67- cells in CITK -/- mice are a mixed population, with only <10% being neurons, and some are PAX6+ and TBR2+. This is confusing, because PAX6+ and TBR2+ should rarely be Ki67-, and all Ki67- cells should be neurons.

**Response:**
The numbers which we mentioned in the rebuttal letter were referred to the Brdu+ Ki67- cells of the VZ/SVZ. In general, also in our samples most Ki67- cells are neurons. In CITK knockout samples we found an increase of Brdu+ Ki67- cells, and only in VZ/SVZ we noted that some of them are Pax6+ or Tbr2+. These cells could be early neuroblasts still expressing progenitor markers or growth arrested progenitors.

**Comment:**
Previous Main Comments Point 4:
The authors could not do the experiment of disrupting ASPM-CITK interaction to assess spindle misorientation, as they claim that these two proteins interact through multiple regions and could not define a single concrete sequence to disrupt the interaction. For the other experiment of targeting CITK with centrosome localization sequence to rescue ASPM phenotype, they cite time restraint for not doing the experiment.

This is one of the most essential experiments to demonstrate the significance of the functional link between ASPM and CITK in spindle orientation. Without this data, the strength of this paper is greatly compromised.

**Response:**
We have decided to include the data of the immunoprecipitation experiments, showing that both the amino- and the carboxi-terminal halves of CITK are capable of interacting with the C-terminal region of ASPM.

3rd Editorial Decision 19 July 2016

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Thank you for your patience while we have reviewed your revised manuscript. As you will see from the report below, referee 3 now supports publication in EMBO reports. I am therefore writing with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once a few minor issues/corrections have been addressed, as follows.

- Regarding data quantification, you have specified the number "n" for how many experiments were performed and the error bars (e.g. SEM, SD) in almost all figure legends except for Fig. 1D, 1F, 5E, EV1D. Could you please add this information? I also noted that the scale bar in Fig. 1A is not labeled. Moreover, the scale bars in Fig. 3 appear very thin and might not be visible in the print version of the manuscript. You might want to make the lines a bit thicker.

- I also noted a mistake in the figure legend of Fig. EV1 (C). Please correct.

If all remaining corrections have been attended to, you will then receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt inclusion of your manuscript in our next available issue.

Thank you for your contribution to EMBO reports.

REFEREE REPORTS

Referee #3:

I am ok with the publication.

3rd Revision - authors' response 22 July 2016

Author made necessary changes.

4th Editorial Decision 26 July 2016

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Ferdinando Di Cunto; Marta Gai
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## Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

### 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 replicates.
- 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 author ship guidelines on Data Presentation.

### 2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

**Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.**

**In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).**

### B- Statistics and general methods

Please fill out these boxes (Do not worry if you cannot see all your text once you press return)

| Question | Answer |
|---|---|
| 1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size? | For the experiments in which we can show statistically significant differences, sample size was determined on the basis of pilot studies estimating the order of magnitude of differences. For the experiments showing non significant differences, we used the same sample size of those revealing differences. (Pag. 20) |
| 1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used. | CITK phenotypes are usually 100% penetrant with minimal variability due to genetic background. Under these conditions, analysis of three biological replicates is usually sufficient to reveal the existence of significant differences (Pag. 16) |
| 2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established? | We usually exclude samples that are not judged of sufficient technical quality. In this study no samples were excluded. (Pag. 17) |
| 3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe. | We use as much as possible automatic quantification tools. When this is not possible, data quantification is performed blind to genotypes/conditions. (Pag 19) |
| For animal studies, include a statement about randomization even if no randomization was used. | Sample size is not big enough to reasonably require randomization (detail not provided in the text) |
| 4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe. | N/A (because we did not use randomization) |
| 4.b. For animal studies, include a statement about blinding even if no blinding was done | Sample preparation and analysis are performed by different operators (Pag. 19) |
| 5. For every figure, are statistical tests justified as appropriate? | We think so |
| Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. | Graphical analysis of data distribution analysis (Pag. 20) |
| Is there an estimate of variation within each group of data? | No |
| Is the variance similar between the groups that are being statistically compared? | Yes |

### C- Reagents

| | |
|---|---|
| 6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right). | Mouse anti-alpha-tubulin, Sigma, Cat. T5168, clone B-5-1-2. Rabbit anti-alpha-tubulin, Abcam, cat. ab15246. Mouse anti-gamma-tubulin, Abcam, clone TU-30,cat. ab27074. Rabbit anti-gamma-tubulin, Sigma, cat. T5192. Mouse anti-CITK, BD Transduction Laboratories, clone 6/CRIK, cat. 611376. Rabbit anti-Numa, Abcam, cat. ab36999. Rabbit anti-Dynein Heavy Chain, Santa Cruz, cat. sc-9115. Mouse anti-p150, BD Biosciences, cat. 610474. Rabbit anti-GFP, Abcam, cat. ab290. Mouse anti-Ki67, BD Biosciences, clone B56, cat. 550609. Mouse anti-BrdU, Chemicon, Cat. MAB-1467. Mouse anti-TUBB3, clone TuJ1, Covance, cat. MMS-435P; Rabbit anti-Tbr2, Millipore, cat. (Pag 16) |
| 7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination. | HeLa cells were originally bought from ATCC and a batch was frozen after 5 passages. Cells are routinely screened for mycoplasma contamination. (Pag 14) |

* for all hyperlinks, please see the table at the top right of the document

## D- Animal Models

| | |
|---|---|
| 8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals. | CITK -/- mouse embryos C57/Bl6 x Sv129, E14.5. Animal were housed in the Animal facility of Molecular Biotechnology Centre, University of Torino. (Pag 16) |
| 9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments. | Italian Ministry of Health, licence n. 343/2015 PR (Pag 16) |
| 10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance. | Confirmed |

## E- Human Subjects

| | |
|---|---|
| 11. Identify the committee(s) approving the study protocol. | N/A |
| 12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report. | N/A |
| 13. For publication of patient photos, include a statement confirming that consent to publish was obtained. | N/A |
| 14. Report any restrictions on the availability (and/or on the use) of human data or samples. | N/A |
| 15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable. | N/A |
| 16. 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 you have submitted this list. | N/A |
| 17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines. | N/A |

## F- Data Accessibility

| | |
|---|---|
| 18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'.<br><br>Data deposition in a public repository is mandatory for:<br>a. Protein, DNA and RNA sequences<br>b. Macromolecular structures<br>c. Crystallographic data for small molecules<br>d. Functional genomics data<br>e. Proteomics and molecular interactions | N/A |
| 19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right). | N/A |
| 20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right). | N/A |
| 21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section.<br><br>Examples:<br>**Primary Data**<br>Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462<br>**Referenced Data**<br>Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26<br>AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208 | N/A |
| 22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information. | N/A |

## G- Dual use research of concern

| | |
|---|---|
| 23. 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 (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could. | NO |