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Review COMMONS

Annexin A2 and Ahnak control cortical NuMA-dynein localization and mitotic spindle orientation

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Reviewer 1

Evidence, reproducibility and clarity

Gallaud et al. report on experiments in human cells identifying previously unrecognized roles of the Annexin2 (Anx2) - Ahnak complex in mitotic spindle positioning. The logic of the story is straight-forward: knockdown of either one gene product leads to mitotic delay in pre-synchronized cells when proceeding through mitosis. This is supposed to be a consequence of defects in spindle position, which is directly shown when measuring spindle angels and spindle rocking in live cells. The two proteins are found in a common complex. Although there is no biochemical evidence for direct interaction, an Anx2 mutant previously shown to fail in Ahnak interaction displays similar phenotypes as the ones observed upon Ahnak or Anx2 knockdown. Non-homogenous, cortical localization of the proteins is consistent with a functional interaction with the Dynein-motor complex. The cortical localization of Dynein, in turn, depends on the localization of Anx2 and Ahnak there.

Taken together, the data are interesting and identify functions for these proteins that have not been described yet may have been expected for these two proteins.

Several experimental issues, however, remain to be solved in order to result in a competitively publishable manuscript:

Major:

1) Statistics in most experiments are rather poor, i.e. the authors show quantifications of small samples sizes and do not indicate the number of replicates (technical/biological).

2) While the authors present one statistically robust rescue experiment (Fig. 1 F), most knockdowns stand alone and are not further substantiated using siRNA-resistant expression of Anx2-GFP. Even for the rescue experiment shown, we neither know transfection

efficiency nor expression levels in individual cells. Why was the stably Anx2-GFP expressing cell line not used to validate the knockdowns?

3) Knockdown delays cells in mitosis, fair enough. Evidence for SAC activation is, however, poor. A couple of cells showing BUBR1 localization does not make the point. What about a co-knockdown of MAD2? This is an important point since delay in early mitosis and SAC activity may well govern the observed phenotypes.

4) The observation that dynein localization is affected is most intriguing. What happens to astral MT in general? Measuring MT dynamics may go beyond an initial characterization but showing astral/cortical MT at reasonable resolution would be very important.

Minor:

5) Interaction studies shown are shown for an interaction of Anx2 and Ahnak and with Dynein. Yet we do not know any details (which domains of the protein are involved in the interaction and is this functionally relevant?). I do concede that the use of the N-terminal mutant of Anx2. Why not e.g. using a truncation of the N-terminus to confirm the result? The IP/pulldown fractions also do not contain any negative control of a non-interacting protein detected by Western blotting.

6) Experiments are only done in pre-synchronized cells released from G2/M arrest. This focused assay certainly makes sense but why not showing the impact on a non-synchronous culture first?

7) Maybe more than a matter of taste to only use HeLa Kyoto cells for all experiments. I strongly suggest confirming at least the initial knockdown phenotypes in less transformed cells such as RPE-1.

Significance

The significance of this study for experts in the field is certainly high. The paper aims at identifying two previously not well recognized proteins in spindle positioning, shows their interaction and demonstrates communication with dynein. However, we miss mechanistic details and therefore new surprising outcomes that would be a true conceptual advancement. On the other hand, the study is logically very clearly structured and certainly worth to be communicated in an internationally visible journal given that the issues raised are, at least to some extent, addressed.

Reviewer 2

Evidence, reproducibility and clarity

Summary:

In this manuscript, Gallaud et al. investigate a role of the Annexin A2(Anx2)/Ahnak complex in mitosis. In previous work, the senior author has demonstrated a role for Anx2 in the Rho-dependent formation of the cleavage furrow during cytokinesis (Benaud et al., Embo Rep 2015). In the current manuscript the authors demonstrate Anx2 is also important during earlier phases of mitosis. Depletion of Anx2 in Hela cells results in mitotic defects, as this strongly delays progression into anaphase, and disrupts spindle orientation that has previously been shown to be instructed by cell-ECM adhesion cues in

Hela cells. The authors show Anx2 is necessary to recruit Ahnak to the cell cortex facing spindle poles.
Furthermore, they propose the Anx2/Ahnak complex controls spindle orientation through an association with the dynein-dynactin complex, which is absent from the cortex near the spindle poles upon Anx2 or Ahnak depletion.

Major comments:

While the observed phenotypes of Anx2/Ahnak depletion are clear, my main concern about this manuscript is that the findings regarding the regulation of Anx2/Ahnak localization as well as their function in mitosis are not conclusive.

- The authors perform a co-immunoprecipitation experiment to show dynein/dynactin associate with Ahnak, but whether this directs the cortical distribution of the dynein complex in mitosis remains speculative. Ahnak depletion also results in reduced lateral oscillations of the mitotic spindle (Fig. 5), and increased distance of the spindle from the cortex acts as a positive signal for dynein/dynactin localization at the cell cortex (Kiyomitsu et al., NCB 2012 and see also Fig. 5c). The authors would have to investigate whether the Anx2/Ahnak complex directly regulates cortical dynein/dynactin distribution, or whether this is an indirect effect (either through spindle oscillations or through regulation of other dynein/dynactin interacting proteins linked to spindle orientation in Hela cells, e.g. LGN/NuMA; JAM-A).
- The authors should test whether cell-ECM adhesion is not affected by the depletion of Anx2/Ahnak, to exclude that this underlies the defect in adhesion-mediated spindle orientation.
- The authors propose that adhesion cues influence the cortical distribution of Anx2/Ahnak (enriched at the cortex adjacent to retraction fibers), however their experiments lack essential controls to conclude this. Is this enrichment of Anx2/Ahnak already apparent before the spindle is formed (or instead of being regulated by adhesion cues, could their enrichment involve signals originating from the spindle)? The authors should therefore test the distribution of Anx2/Ahnak upon disruption of spindle formation (e.g. nocodazole treatment). Importantly, all localization experiments should be controlled by comparing the distribution of Anx2/Ahnak to control proteins (e.g. CAAX-GFP) that are expected to localize uniformly at the cortex, and the enrichment of Anx2/Ahank should be analyzed relative to these control proteins. This should be done in in more than 1 example cell as is done now, in particular because in several examples it is difficult to see any enrichment at the cortex near the spindle poles (e.g. Anx2-Gfp in Fig. 3D).
- The authors imply a role for cortical Ahnak in regulating anchoring of the mitotic spindle and consequently spindle orientation and anaphase delay. The data supporting this is not convincing, in particular because Ahnak depletion results in several other effects that may explain these phenotypes: for instance, the disruption of spindle morphology as shown in Fig. 4D that may impact mitotic progression. Therefore, whether attenuated cortical force generation underlies the observed phenotypes as proposed by the authors remains entirely speculative. In addition, while the authors base a role for cortical Ahnak in part on the S100A2-binding mutant of Anx2, this mutant will generally affect formation of this complex and not only disrupt cortical Ahnak localization.
- Does Anx2 remain present at the cell cortex upon Ahnak depletion?
- While the number of cells that is analyzed is indicated, it is unclear from how many independent experiments these analysed cells were derived, and if three independent experiments have been performed for all the shown data (and therefore it is also unclear how statistical analyses were performed).
- The presence of cell neighbors will influence several of the parameters analyzed throughout the manuscript (e.g. protein localization, see Fig. 3G). The authors should be consistent in their analyses with including/excluding cells in contact with neighbors.

Minor comments:

- Fig. 2F showing cortical localization of Anx2 during mitosis should also include an image of the same cell in interphase (and the same should be shown for Ahnak).
- The authors should also show spindle morphology upon Anx2 depletion, as shown for Ahnak in Fig. 4D.
- The authors should indicate more clearly how analyses were performed; e.g. when spindle angles where measured (in Figs. 2C-E, 4C) and whether this included Anx2/Ahnak depleted cells stuck in prometaphase, and describe more clearly how the distribution of proteins was measured.
- Fig. 2A is a bit difficult to understand, in particular because of the dotted lines. The authors may consider rephrasing the explanation of this figure.
- It remains unclear throughout the manuscript how the different phenotypes (delay anaphase onset and spindle misorientation) are connected, and whether the authors think one may be causal for the other that they completely independent of each other.
- It may be interesting to investigate whether Anx2/Ahnak specifically function in spindle orientation instructed by cell-ECM adhesion cues (as in Hela cells), or fulfil a more general in spindle orientation. For instance, the authors could investigate whether Anx2/Ahnak also functions in planar divisions or epithelial cells that are directed by cell-cell adhesion cues.

Significance

This manuscript adds to the existing knowledge on spindle orientation induced by cell-matrix adhesion cues, as it demonstrates an essential role for the Anx2/Ahnak complex in this process. Furthermore, it demonstrates a role for this complex in anaphase progression. However, the exact function of Anx2/Ahnak in both of these processes remains unclear and would require further investigation. These findings will be relevant to an audience interested in understanding the molecular mechanisms underlying mitotic spindle regulation and the organization of the mitotic cell cortex.

Field of expertise: cell division orientation, cell adhesion.

Reviewer 3

Evidence, reproducibility and clarity

Summary

Benaud and colleagues have previously shown that Anx2 plays role in epithelial cell polarization and in cytokinesis (Benaud et al., JCB 2004; Benaud et al., EMBO Rep 2015). In this study, authors focus on the role of Anx2 in mitosis. After siRNA-mediated depletion of Anx2, they observed impaired progression through mitosis and delayed anaphase onset. In addition, Anx2 depletion increased the angle between the spindle axis and slide surface suggesting that Anx2 is needed for proper orientation of the mitotic spindle. Importantly, expression of Anx2-GFP rescued the mitotic delay as well as the spindle orientation defect validating the specificity of the siRNA. Anx2 was enriched in cell cortex region close to the spindle poles in metaphase and this localization was influenced by cell adhesion to fibronectin. Immunoprecipitation assays showed that similarly as in interphase, Anx2 interacted with S100A10 and Ahnak also during mitosis. Further, authors inserted GFP tag in frame with endogenous Ahnak and observed that Ahnak localized at the retracting cortex in prometaphase. Later it colocalized with Anx2 at the cortex close to the spindle poles. Upon depletion of Anx2, accumulation of Ahnak-GFP at the retracting cell cortex

was reduced. Similarly, Ahnak-GFP localization was affected by expression of a dominant negative mutant of Anx2. Depletion of Ahnak by siRNA showed similar mitotic phenotypes as depletion of Anx2. Depletion of Ahnak and/or Anx2 impaired localization of GFP-dynein to the cell cortex and impaired lateral oscillations during metaphase. Authors conclude that Anx2 and Ahnak regulate mitotic spindle orientation by controlling localization of dynein to cell cortex.

Overall, I find the study well performed and the results are convincing. Some exceptions are specified below.

Major comments

Authors speculate that Anx2/Ahnak-dependent control of dynein localization may have evolved in parallel with the LGN/NuMA pathway. The study would benefit from showing whether manipulation of Anx2/Ahnak influences localization of LGN and NuMA in metaphase. Co-depletion of Anx2 and NuMA may also help to show if both proteins act in parallel pathways.

Minor comments

1. How many cells were quantified in Fig. 2G and 5B? More than one cell need to be analyzed to support the conclusions.
2. Fig 2H does not seem to support the asymmetric localization of endogenous Anx2 to polar cortex during metaphase. More cells and quantification is needed.
3. It is unclear how authors quantified enrichment of Ahnak-GFP in cell cortex in Fig. 3E. Design of the figure should be unified with that in Fig 2I. Number of quantified cells should be stated.
4. Authors state that dynein localization at kinetochore is not affected by Ahnak or Anx2 knock down but this is not visible in Fig. 5C.
5. Authors should quantify chromosomal alignments after knock down of Ahnak or Anx2
6. Fixation protocol with TCA should be described in methods. To allow reproducibility, figure legend should specify which fixation method was used (methanol or TCA).

Significance

Description of the Anx2 function in spindle positioning is novel and provides conceptual advance to the mitotic research. Some limitation of the study is a lack of mechanistic insight on function of the giant Ahnak protein. On the other hand, the study opens new exciting questions to the field. In the future, it would be interesting the address the role of phosphatidylinositols in organization of the cortical compartment during mitosis. Does Annexin2 localization depend on its ability to bind PIPs? Interestingly, NuMA has also been reported to bind PIP2 (Kotak et al., EMBO J 2014) that I believe should be discussed.

Author response to reviewers' comments

Revision Plan:

All three reviewers have pointed out the originality and significance of this study in the field of spindle orientation and mitosis. We believe that we can fully address the issues raised by the reviewers. We will improve the clarity of the manuscript and perform the experiments suggested by the reviewers. Below is the point by point revision plan to improve the manuscript.

Reviewer 1:

1) Statistics in most experiments are rather poor, i.e. the authors show quantifications of small samples sizes and do not indicate the number of replicates (technical/biological).
The number of replicates and cells analyzed will be indicated in each figure and the Material and Method section amended. New acquisitions will be performed to improve the statistics and we will present the new data when needed. However, it is often complicated to reach high number of mitotic cells that can be properly analyzed when grown on micropatterns and acquisition performed at high magnification (60x).

2) While the authors present one statistically robust rescue experiment (Fig. 1 F), most knockdowns stand alone and are not further substantiated using siRNA-resistant expression of Anx2-GFP. Even for the rescue experiment shown, we neither know transfection efficiency nor expression levels in individual cells. Why was the stably Anx2-GFP expressing cell line not used to validate the knockdowns?
The Anx2-GFP siRNA resistant cell line was shown in a previous study to rescue the late cell division defects following Anx2 knockdown (Benaud *et al.*, EMBO Rep 2015). In this study, as noticed by the reviewer, we also show that the siRNA resistant construct rescues the mitotic delay following endogenous depletion of Anx2 (Fig1F). Moreover, the spindle orientation defect is also fully rescued (Fig. 2E). These experiments indicate that the construct is fully functional. We will perform and provide in the revised version additional phenotypes rescue.

3) Knockdown delays cells in mitosis, fair enough. Evidence for SAC activation is, however, poor. A couple of cells showing BUBR1 localization does not make the point. What about a co-knockdown of MAD2? This is an important point since delay in early mitosis and SAC activity may well govern the observed phenotypes.
We agree and will perform more experiments in order to provide robust evidence of SAC activation.

4) The observation that dynein localization is affected is most intriguing. What happens to astral MT in general? Measuring MT dynamics may go beyond an initial characterization but showing astral/cortical MT at reasonable resolution would be very important.
As suggested, we will look at the astral MTs and provide astral/cortical MT quantifications in a new figure.

Minor:
5) Interaction studies shown are shown for an interaction of Anx2 and Ahnak and with Dynein. Yet we do not know any details (which domains of the protein are involved in the interaction and is this functionally relevant?). I do concede that the use of the N-terminal mutant of Anx2. Why not e.g. using a truncation of the N-terminus to confirm the result? The IP/pulldown fractions also do not contain any negative control of a non-interacting protein detected by Western blotting.
A negative control will be provided

6) Experiments are only done in pre-synchronized cells released from G2/M arrest. This focused assay certainly makes sense but why not showing the impact on a non-synchronous culture first?
Experiments presented in Fig. 1 C,D and Fig. 2 A,B were performed in a non-synchronized cell population. We will clarify this point in the figure legend.

7) Maybe more than a matter of taste to only use HeLa Kyoto cells for all experiments. I strongly suggest confirming at least the initial knockdown phenotypes in less transformed cells such as RPE-1.
We will confirm the initial knock down phenotype of spindle orientation defect on RPE cells.

Reviewer 2:

- The authors perform a co-immunoprecipitation experiment to show dynein/dynactin associate with Ahnak, but whether this directs the cortical distribution of the dynein complex in mitosis remains speculative. Ahnak depletion also results in reduced lateral oscillations of the mitotic spindle (Fig. 5), and increased distance of the spindle from the cortex acts as a positive signal for dynein/dynactin localization at the cell cortex (Kiyomitsu et al., NCB 2012 and see also Fig. 5c). The authors would have to investigate whether the Anx2/Ahnak complex directly regulates cortical

dynein/dynactin distribution, or whether this is an indirect effect (either through spindle oscillations or through regulation of other dynein/dynactin interacting proteins linked to spindle orientation in Hela cells, e.g. LGN/NuMA; JAM-A).
This is a good suggestion and we will investigate whether manipulation of Anx2 /Ahnak influence the localization of NuMa and LGN.

- The authors should test whether cell-ECM adhesion is not affected by the depletion of Anx2/Ahnak, to exclude that this underlies the defect in adhesion-mediated spindle orientation.
Note that our experiments presented Fig. 2K, Fig. 3E were performed with cells adherent on fibronectin coated micropatterns. Clearly, cells depleted with annexin 2 are still able to attach, spread on fibronectin coated micropatterns and progress thru mitosis.
To address this important point, we test whether annexin 2 and ahnak depleted cells retain the ability to adhere and fully spread on fibronectin-coated glass coverslips. This result will be discussed to strengthen the manuscript.

- The authors propose that adhesion cues influence the cortical distribution of Anx2/Ahnak (enriched at the cortex adjacent to retraction fibers), however their experiments lack essential controls to conclude this. Is this enrichment of Anx2/Ahnak already apparent before the spindle is formed (or instead of being regulated by adhesion cues, could their enrichment involve signals originating from the spindle)? The authors should therefore test the distribution of Anx2/Ahnak upon disruption of spindle formation (e.g. nocodazole treatment).
Anx2 and Ahnak are both enriched adjacent to retractation fibers before the mitotic spindle is fully formed. The localization of anx2-GFP from cells plated on fibronectin coted L-shape patterns in prophase, before nuclear envelop break down and spindle formation, will be provided. In addition, we will also present in the revised version the localization of ahnak and that of ERM (at mitotic entry before the spindle is fully formed.
Last, we will challenge the contribution of spindle microtubules on the mitotic distribution of Ahnak-GFP and Anx 2-GFP. We will provide new experiments showing their localization upon nocodazole mediated disruption of the mitotic spindle.
Importantly, all localization experiments should be controlled by comparing the distribution of Anx2/Ahnak to control proteins (e.g. CAAX-GFP) that are expected to localize uniformly at the cortex, and the enrichment of Anx2/Ahank should be analyzed relative to these control proteins. This should be done in in more than 1 example cell as is done now, in particular because in several examples it is difficult to see any enrichment at the cortex near the spindle poles (e.g. Anx2-Gfp in Fig. 3D).
We understand the reviewer's concern. We will provide the quantification of the cortical distribution of Anx2-GFP relative to MyrPalm-mCherry as a uniform cortical localization control. Since Anx2 localization is not restricted to the cortex and a cytoplasmic population is also present, in fixed samples the optimal visualization of the cortical signal requires higher resolution image. In the revised version we will thus provide a better resolution image for Fig 3D.

- The authors imply a role for cortical Ahnak in regulating anchoring of the mitotic spindle and consequently spindle orientation and anaphase delay. The data supporting this is not convincing, in particular because Ahnak depletion results in several other effects that may explain these phenotypes: for instance, the disruption of spindle morphology as shown in Fig. 4D that may impact mitotic progression. Therefore, whether attenuated cortical force generation underlies the observed phenotypes as proposed by the authors remains entirely speculative. In addition, while the authors base a role for cortical Ahnak in part on the S100A2-binding mutant of Anx2, this mutant will generally affect formation of this complex and not only disrupt cortical Ahnak localization.
We will revise our discussion and develop it to address this important point. We will focus our discussion to the role of the anx2/ahnak complex in guiding spindle orientation in response to adhesion cues and in promoting the cortical polarization of the cortical force elements. As the reviewer pointed out, at this point we cannot exclude that the delay in metaphase onset reflecting spindle–checkpoint mediated activation may be induced by additional defects. Indeed, we cannot rule out a direct effect of Anx2 and Ahnak on spindle assembly. In addition, the use of the S100A2-binding mutant was to show, using a different technique that RNAi, the importance of the Anx2 for spindle orientation. This mutant indeed impairs Anx2 function, not only Ahnak localization.
The result and the discussion will be modified accordingly to this reviewer's suggestion.

- Does Anx2 remain present at the cell cortex upon Ahnak depletion?
Co-dependence of anx2 and ahnak cortical localization will be investigated in a revised version.

- While the number of cells that is analyzed is indicated, it is unclear from how many independent experiments these analysed cells were derived, and if three independent experiments have been performed for all the shown data (and therefore it is also unclear how statistical analyses were performed).
The experiments have been repeated according to the standard in the field and we will modify the figure legend and the Material and Method section according to the reviewer request.

- The presence of cell neighbors will influence several of the parameters analyzed throughout the manuscript (e.g. protein localization, see Fig. 3G). The authors should be consistent in their analyses with including/excluding cells in contact with neighbors.
We will exclude cell to cell contact and replace panel 3G.

Minor comments:*

- Fig. 2F showing cortical localization of Anx2 during mitosis should also include an image of the same cell in interphase (and the same should be shown for Ahnak).
We will provide images of Anx2-GFP and Ahnak-GFP in interphase cells. We did already provide ahnak-GFP cells adhering to L shape micropatterns before mitotic entry in Fig 3E. We will add a similar panel in Fig. 2I for anx2-GFP.

- The authors should also show spindle morphology upon Anx2 depletion, as shown for Ahnak in Fig. 4D.
We will add the spindle morphology upon Anx2 depletion in Fig 4D.

- The authors should indicate more clearly how analyses were performed; e.g. when spindle angles where measured (in Figs. 2C-E, 4C) and whether this included Anx2/Ahnak depleted cells stuck in prometaphase, and describe more clearly how the distribution of proteins was measured.
We will revise the Material and Method section to explain in more details how the analysis was performed for Fig 2C-E, Fig 4C.

- Fig. 2A is a bit difficult to understand, in particular because of the dotted lines. The authors may consider rephrasing the explanation of this figure.
We will rephrase the figure legend for Fig. 2A to clarify it.

- It remains unclear throughout the manuscript how the different phenotypes (delay anaphase onset and spindle misorientation) are connected, and whether the authors think one may be causal for the other that they completely independent of each other.
We will develop this point in the discussion.

- It may be interesting to investigate whether Anx2/Ahnak specifically function in spindle orientation instructed by cell-ECM adhesion cues (as in Hela cells), or fulfil a more general in spindle orientation. For instance, the authors could investigate whether Anx2/Ahnak also functions in planar divisions or epithelial cells that are directed by cell-cell adhesion cues.
Indeed, it is an interesting point. We believe that Ahnak could play a more general role in spindle orientation, albeit some differences could be present depending on the cellular context. For sake of clarity, we believed we should study independently adhesion cue and cell-cell adhesion cues. We decided to focus on adhesion-dependent cue and isolated cells for the purpose of this study. However, we will comment this interesting point in the discussion.

<u>Reviewer 3:</u>

Major comments
Authors speculate that Anx2/Ahnak-dependent control of dynein localization may have evolved in parallel with the LGN/NuMA pathway. The study would benefit from showing whether manipulation of Anx2/Ahnak influences localization of LGN and NuMA in metaphase. Co-depletion of Anx2 and NuMA may also help to show if both proteins act in parallel pathways.

This is an interesting point, the contribution of Anx2 /Ahnak influence for the localization of NuMa and LGN will be presented in a revised version.

Minor comments

1. How many cells were quantified in Fig. 2G and 5B? More than one cell need to be analyzed to support the conclusions.

The experiments have been repeated according to the standard in the field and we will modify the figure legend according to the reviewer requested. We will provide the quantifications for Fig 2G and 5B and indicate the number of cells analyzed and the number of replicates performed.

2. Fig 2H does not seem to support the asymmetric localization of endogenous Anx2 to polar cortex during metaphase. More cells and quantification is needed.

Note that Fig 2H is a fixed IF of endogenous annexin 2. We are limited by the availability of antibodies that function in immunofluorescence under conditions that preserve cortical localization. After several attempt, we were able to detect Anx2 on the polar cortex but the images are less qualitative that those obtained using the functional Anx-GFP functional transgene. We will provide a higher resolution image as well as quantification.

3. It is unclear how authors quantified enrichment of Ahnak-GFP in cell cortex in Fig. 3E. Design of the figure should be unified with that in Fig 2I. Number of quantified cells should be stated.

In Fig. 3E, we will state the number of cells analyzed and unify the figure as well as the quantification with Fig. 2I

4. Authors state that dynein localization at kinetochore is not affected by Ahnak or Anx2 knock down but this is not visible in Fig. 5C.

We will rephrase our statement to be correct by « we could still observe some dynein recruitment at the kinetochore » and provide as supplementary panel the full image corresponding to the kymograph illustrating kinetochore recruitment.

5. Authors should quantify chromosomal alignments after knock down of Ahnak or Anx2

As requested, we will quantify the chromosome alignment after Ahnak and Anx2 knock down

6. Fixation protocol with TCA should be described in methods. To allow reproducibility, figure legend should specify which fixation method was used (methanol or TCA).

We will describe the TCA fixation protocol in the method section and indicate the fixation used in the figure legends.

Does Annexin2 localization depend on its ability to bind PIPs? Interestingly, NuMA has also been reported to bind PIP2 (Kotak et al., EMBO J 2014) that I believe should be discussed.

We agree this is an interesting point and will discuss it in the revised version.

Original submission

First decision letter

MS ID#: JOCES/2021/259344

MS TITLE: Annexin A2 and Ahnak control cortical dynein localization and mitotic spindle orientation

AUTHORS: Emmanuel Gallaud, Aude Pascal, Regis Giet, and Christelle Benaud
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

As the reviewers raise a number of criticisms, I cannot accept the paper at this stage. If you address their concerns along the lines you indicate, then I would be pleased to see a revised manuscript. We would then return it to the reviewers.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

First revision

Author response to reviewers' comments

Revision:

Below is the point by point response to the reviewers indicating the changes performed in the revised manuscript.

Reviewer 1:

1) Statistics in most experiments are rather poor, i.e. the authors show quantifications of small samples sizes and do not indicate the number of replicates (technical/biological).

In order to improve the statistics, we have now performed new acquisitions to increase the sample sizes for all the quantifications performed with the smallest samples. We are now presenting new data for Fig. 2B (for sake of visibility Fig. 2A was not increased since Fig. 2B was improved), Fig. 2I, 2J, Fig. 3I, 3L. For live imaging on patterns at high magnification (60x), technical limitations and limited access to the pattern chips are explaining the lower number of cells that we have analyzed. We have now ensured that the number is >20 cells.

Additional quantifications have also been performed and are presented: Fig. 1G, Fig. 2H, Fig. 3F, Fig. 3H, Fig. 5E. We have also indicating the number of replicates performed for all the experiments. We are providing data from at least three independent experiments for all of the quantifications.

2) While the authors present one statistically robust rescue experiment (Fig. 1 F), most knockdowns stand alone and are not further substantiated using siRNA-resistant expression of Anx2-GFP. Even for the rescue experiment shown, we neither know transfection efficiency nor expression levels in individual cells. Why was the stably Anx2-GFP expressing cell line not used to validate the knockdowns?

Anx2 rescue experiments are shown for both anaphase transition delay in Fig. 1 (Fig. 1F) and spindle orientation defect in Fig. 2 (Fig. 2E) together with the results from two independent siRNA. Albeit no rescue is provided for experiments performed on patterns Fig. 2K and Fig. 3I, the rescue of spindle orientation has been performed on non-patterned cells (Fig 2E). Live rescue is not possible for dynein localization (Fig5) because both dynein HC and Anx2 are tagged with GFP. We have generated a mcherry and a scarlett tag version of the Anx2, but those tags impact Anx2 cortical localization. In Fig 5, we have now performed an additional rescue experiments for NuMa localization and are presenting the results in Fig. 5J.

3) Knockdown delays cells in mitosis, fair enough. Evidence for SAC activation is, however, poor. A couple of cells showing BUBR1 localization does not make the point. What about a co-knockdown of MAD2? This is an important point since delay in early mitosis and SAC activity may well govern the observed phenotypes.

We agree more experiments were required to provide a more convincing evidence of SAC activation. We have now included a quantification for BUBR1 localization in Fig. 1G. We also provide an additional experiment showing that the Mps1kinase inhibitor abolishes the mitotic delay induced by Anx2 depletion (Fig. 1 H, I.; text p4).

4) The observation that dynein localization is affected is most intriguing. What happens to astral MT in general? Measuring MT dynamics may go beyond an initial characterization but showing astral/cortical MT at reasonable resolution would be very important.
As suggested, we have looked at the astral MTs. Our results indicate that depletion of Anx2 and Ahnak result in a decrease in astral MTs intensity. We are now providing astral/cortical MT quantifications in a new figure (Fig. 4 E, F). These results are presented (p.7) and discussed (p.10).

Minor:
5) Interaction studies shown are shown for an interaction of Anx2 and Ahnak and with Dynein. Yet we do not know any details (which domains of the protein are involved in the interaction and is this functionally relevant?). I do concede that the use of the N-terminal mutant of Anx2. Why not e.g. using a truncation of the N-terminus to confirm the result? The IP/pulldown fractions also do not contain any negative control of a non-interacting protein detected by Western blotting.

We are now providing a negative control for the IP and show that tubulin does not co-IP under our experimental condition. We believe that identifying the domain of interaction is a challenging task that is beyond the scope of the initial publication. Indeed, to date only one protein (Ahnak) has been identified to directly interact with Anx2 and the domain of interaction is not linear (Ozorowski *et al* Acta Crystallogr. 2013). Based on the present results, we cannot exclude the presence of a larger complex and thus are no claiming for a direct interaction. We have now added this point in the discussion p10.

6) Experiments are only done in pre-synchronized cells released from G2/M arrest. This focused assay certainly makes sense but why not showing the impact on a non-synchronous culture first?
Experiments presented in Fig. 1C, D, H and Fig. 2 A, B were performed in a non- synchronized cell population. We have now clarified this point in the figure legend.

7) Maybe more than a matter of taste to only use HeLa Kyoto cells for all experiments. I strongly suggest confirming at least the initial knockdown phenotypes in less transformed cells such as RPE-1.
We have now confirmed the knock down phenotype of Ahnak on spindle orientation in MDCK cells, which are dog kidney cells that retain the capacity to polarize in culture. Indeed, we observed similar orientation defect to those we are describing for HeLa cells. MDCK cells being of a different species, only the siRNA directed against Ahnak worked efficiently in these cells, we thus focus on the phenotype induced by Ahnak down regulation. Results are presented in a new figure (Fig S3, text p7).

Reviewer 2:

- The authors perform a co-immunoprecipitation experiment to show dynein/dynactin associate with Ahnak, but whether this directs the cortical distribution of the dynein complex in mitosis remains speculative. Ahnak depletion also results in reduced lateral oscillations of the mitotic spindle (Fig. 5), and increased distance of the spindle from the cortex acts as a positive signal for dynein/dynactin localization at the cell cortex (Kiyomitsu et al., NCB 2012 and see also Fig. 5c). The authors would have to investigate whether the Anx2/Ahnak complex directly regulates cortical dynein/dynactin distribution, or whether this is an indirect effect (either through spindle oscillations or through regulation of other dynein/dynactin interacting proteins linked to spindle orientation in Hela cells, e.g. LGN/NuMA; JAM-A).
We have now investigated whether manipulation of Anx2 /Ahnak influence the localization of NuMA and are now presenting the result in Fig. 5 I and J; text p8/9. Our results indicate that depletion of Anx2/Ahnak alters cortical NuMA localization. We are now addressing these new results in the Discussion section.

- The authors should test whether cell-ECM adhesion is not affected by the depletion of Anx2/Ahnak, to exclude that this underlies the defect in adhesion-mediated spindle orientation.
To address this question, we have performed a new experiment. We have tested whether Anx2 and Ahnak depleted cells retain the ability to adhere and fully spread on fibronectin- coated glass coverslips. This experiment is now presented in Fig. S1D. Note that our experiments presented Fig. 2K and Fig. 3E were performed with cells adherent on fibronectin coated micropatterns. Clearly, cells depleted for Anx2 are still able to attach, spread on fibronectin coated micropatterns and remained attached as they progressed thru mitosis.

The authors propose that adhesion cues influence the cortical distribution of Anx2/Ahnak (enriched at the cortex adjacent to retraction fibers), however their experiments lack essential controls to conclude this. Is this enrichment of Anx2/Ahnak already apparent before the spindle is formed (or instead of being regulated by adhesion cues, could their enrichment involve signals originating from the spindle)? The authors should therefore test the distribution of Anx2/Ahnak upon disruption of spindle formation (e.g. nocodazole treatment).
We are now presenting additional data and have performed a new experiment to provide the additional controls requested by the reviewer. First, we now show the full sequence of Anx2 localization in annexin 2-GFP cells plated on fibronectin coated L-shape patterns starting in prophase before nuclear envelop break down Fig. S1 A. These data show that the enrichment of Anx2 at the cortex facing the adhesion sites is apparent before the spindle is fully formed. We now also show that Ahnak localize at the cortex adjacent to retraction fibers in early prometaphase, before the spindle is fully formed (Fig. S1 B). Last, as suggested by the reviewer, we have examined the mitotic distribution of Ahnak-GFP and Anx 2-GFP upon nocodazole disruption of the mitotic spindle formation in a new experiment (Fig. S1 C, text p6). Our results indicate that the mitotic spindle is not required for the cortical localization of Ahnak and Anx2. However, we are also discussing the possibility of mitotic spindle involvement in modulating their cortical localization (Discussion section p10).

Importantly, all localization experiments should be controlled by comparing the distribution of Anx2/Ahnak to control proteins (e.g. CAAX-GFP) that are expected to localize uniformly at the cortex, and the enrichment of Anx2/Ahank should be analyzed relative to these control proteins. This should be done in in more than 1 example cell as is done now, in particular because in several examples it is difficult to see any enrichment at the cortex near the spindle poles (e.g. Anx2-Gfp in Fig. 3D).
We have now performed new experiments and are providing the localization of the control protein MyrPalm-GFP together with the localization of endogenous Anx2 (Fig. 2 L), and endogenous Ahnak (Fig. 3 D). We are also now quantifying the cortical distribution of Anx2- GFP (Fig. 2 H) and Ahnak localization (Fig. 3 F). Since the distribution of MyrPalm-GFP did not always appeared homogenous throughout the cortex, we decided to provide the ratio of Anx2 or Ahnak intensity (cortex/cytoplasm), as commonly used in similar type of publication (Kotak *et al.*, Embo J. 2014; Matthews *et al.* Dev. Cell 2012; Kschonsak *et al.* J. Cell Science 2018). We are also now showing higher resolution confocal images and replaced previous Fig. 2 H and Fig. 3 D by novel figures Fig. 2 L and Fig. 3E respectively.

- The authors imply a role for cortical Ahnak in regulating anchoring of the mitotic spindle and consequently spindle orientation and anaphase delay. The data supporting this is not convincing, in particular because Ahnak depletion results in several other effects that may explain these phenotypes: for instance, the disruption of spindle morphology as shown in Fig. 4D that may impact mitotic progression. Therefore, whether attenuated cortical force generation underlies the observed phenotypes as proposed by the authors remains entirely speculative. In addition, while the authors base a role for cortical Ahnak in part on the S100A2-binding mutant of Anx2, this mutant will generally affect formation of this complex and not only disrupt cortical Ahnak localization.
This is indeed a good point that has now been discussed in the revised version of this article. We cannot at this point fully exclude that Ahnak also plays a more direct function on spindle assembly, participating to the SAC-dependent mitotic delay we observe. Similar mitotic delay, associated with spindle assembly and orientation defects has been observed after interference with microtubule-associated proteins such as NuMa and the Dynein/Dynactin complex (For review di Pietro *et al.* EMBO reports 2016). However, in contrast to these proteins, Ahnak localization

appears to be restricted to the cell cortex and not to the spindle or kinetochores. Therefore, we rather favor the hypothesis that impairment of MT/cortical interaction can trigger spindle assembly defects and the SAC activation. Further studies would be required to demonstrate whether Ahnak is able to trigger mitotic defects independently of cortical/MT interactions.

- Does Anx2 remain present at the cell cortex upon Ahnak depletion?

This is an interesting point that has been addressed in this revised version of the manuscript. We have now investigated the effect of Ahnak depletion on Anx2 localization. An additional figure is presented in Fig. S2. Results indicates that down regulation of Ahnak results in a decreased in intensity of cortical Anx2. These new results are described in the text and discussed.

- While the number of cells that is analyzed is indicated, it is unclear from how many independent experiments these analysed cells were derived, and if three independent experiments have been performed for all the shown data (and therefore it is also unclear how statistical analyses were performed).

According to the standard in the field at least three independent experiments have been performed for all experiments. In the revised version of the manuscript, we have clarified in the figure legend the number of independent experiments performed. The Material and Method has also been amended.

- The presence of cell neighbors will influence several of the parameters analyzed throughout the manuscript (e.g. protein localization, see Fig. 3G). The authors should be consistent in their analyses with including/excluding cells in contact with neighbors.

When examining the localization of Anx2 and Ahnak in HeLa cells, we have excluded cell to cell contacts. We have now ensured to be consistent in Fig. 3 K, L and replaced consequently the illustration for Fig. 3K (previously Fig. 3G).

Minor comments:

- Fig. 2F showing cortical localization of Anx2 during mitosis should also include an image of the same cell in interphase (and the same should be shown for Ahnak).

We are now providing images of Anx2-GFP and Ahnak-GFP in interphase cells in Fig. 2I (previously Fig. 2 F) and Fig. 3G respectively.

- The authors should also show spindle morphology upon Anx2 depletion, as shown for Ahnak in Fig. 4D.

We have now performed the time-lapse analysis of the mitotic spindle upon Anx2 siRNA treatment. The corresponding time serie has now been added in Fig. 4D.

- The authors should indicate more clearly how analyses were performed; e.g. when spindle angles where measured (in Figs. 2C-E, 4C) and whether this included Anx2/Ahnak depleted cells stuck in prometaphase, and describe more clearly how the distribution of proteins was measured.

We have now rephrased and completed the figure legends to explain more clearly the quantification, including for Fig 2C-E, Fig 4C. Two sections in the Material and Method on how the analyses were performed have now also been included. In addition, we have added a diagram in Fig 2H to clarify how the distribution of the protein was quantified.

- Fig. 2A is a bit difficult to understand, in particular because of the dotted lines. The authors may consider rephrasing the explanation of this figure.

We have now rephrased the figure legend for Fig. 2A to clarify it.

- It remains unclear throughout the manuscript how the different phenotypes (delay anaphase onset and spindle misorientation) are connected, and whether the authors think one may be causal for the other that they completely independent of each other.

We have now fully revised our discussion. We specifically discuss how the different phenotypes can be connected.

- It may be interesting to investigate whether Anx2/Ahnak specifically function in spindle orientation instructed by cell-ECM adhesion cues (as in Hela cells), or fulfil a more general in

spindle orientation. For instance, the authors could investigate whether Anx2/Ahnak also functions in planar divisions or epithelial cells that are directed by cell-cell adhesion cues.
Indeed, it is an interesting point. We believe that Ahnak could play a more general role in spindle orientation. Our data in MDCK cells presented in Fig. S3 indicate that Ahnak localization closely follows that of the cortical anchoring complex at the cortical lateral domain (Busson *et al*. Curr. Biol.1998, di Pietro *et al*. EMBO Rep 2016) and its downregulation alters spindle orientation, pointing to a potential role in planar division. However, these initial results should be further investigated in the context of acini formation in Matrigel. This would be beyond the scope of this study. For sake of clarity, we believed adhesion cue and cell-cell adhesion cues should be studied independently. We decided to concentrate on adhesion-dependent cue and isolated cells for the purpose of this study.

Reviewer 3:

Major comments
Authors speculate that Anx2/Ahnak-dependent control of dynein localization may have evolved in parallel with the LGN/NuMA pathway. The study would benefit from showing whether manipulation of Anx2/Ahnak influences localization of LGN and NuMA in metaphase. Co-depletion of Anx2 and NuMA may also help to show if both proteins act in parallel pathways.

We have now investigated whether manipulation of Anx2 /Ahnak influence the localization of NuMA. The results of these new experiments are now presented in Fig. 5 I, J; text p9. Our results indicate that depletion of Anx2/Ahnak alters cortical NuMA localization. In view of these results we have now adjusted our entire discussion.

Minor comments
1. How many cells were quantified in Fig. 2G and 5B? More than one cell need to be analyzed to support the conclusions.
We are now providing quantifications for Fig. 2G in a new panel Fig. 2H. The quantification corresponding to the phenotype illustrated in Fig. 5B is presented in Fig. 5D. We now clearly indicate the number of cells analyzed and the number of replicates performed in the figure legend. The Material and Method has also been amended.

2. Fig 2H does not seem to support the asymmetric localization of endogenous Anx2 to polar cortex during metaphase. More cells and quantification is needed.
We have now replaced Fig. 2H with Fig. 2L which shows a higher resolution confocal image. This new panel illustrates the asymmetric localization of Anx2. In addition, new quantifications have been performed and a new panel 2H has been added to the figure.

3. It is unclear how authors quantified enrichment of Ahnak-GFP in cell cortex in Fig. 3E. Design of the figure should be unified with that in Fig 2I. Number of quantified cells should be stated.
As requested, to unify the quantification of Fig. 3G (previously Fig 3E) with Fig. 2J (previously Fig. 2I), we have now performed a new quantification presented in panel 3H. For sake of clarity, we have modified the figure legend of Fig. 3I and added precisions on the quantification in the text p7. The number of cells analyzed is now stated in the figure legend. The Material and Method has also been amended.

4. Authors state that dynein localization at kinetochore is not affected by Ahnak or Anx2 knock down but this is not visible in Fig. 5C.
We have now rephrased our statement to be correct by « Dynein targeting to the kinetochore could still be observed under either Ahnak or Anx2 knockdown conditions» (p8). The recruitment of dynein to the kinetochore under Ahnak and Anx2 knockdown condition is visible in the supplementary movies (Movie S1, S2, S3) that provides the full size, time-lapse images corresponding to the Fig. 5C kymographs.

5. Authors should quantify chromosomal alignments after knock down of Ahnak or Anx2 As requested, we have now quantified chromosome alignment in control, Ahnak and Anx2 knock down mitotic cells. A new panel has now been added in Figure 5, Fig. 5E and the results presented in the text.

6. Fixation protocol with TCA should be described in methods. To allow reproducibility, figure legend should specify which fixation method was used (methanol or TCA).
We are now describing the TCA fixation protocol in the method section and have indicated when this fixation is used.

Does Annexin2 localization depend on its ability to bind PIPs? Interestingly, NuMA has also been reported to bind PIP2 (Kotak et al., EMBO J 2014) that I believe should be discussed. We agree this is an interesting point that is now discussed in this revised version (p10-11).

Second decision letter

MS ID#: JOCES/2021/259344

MS TITLE: Annexin A2 and Ahnak control cortical NuMA-dynein localization and mitotic spindle orientation

AUTHORS: Aude Pascal, Emmanuel Gallaud, Regis Giet, and Christelle Benaud
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers gave favourable reports but raised some critical points that will require amendments to your manuscript. Reviewer 2 offers some extensive comments, which I believe can be addressed in the text without requiring further expeirments. Please indicate to me how you have responded to these points in your rebuttal letter. I hope that you will be able to carry out these revisions because I would like to be able to accept your paper.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In the revised manuscript, authors provide new data that support their former conclusion that Anx2 controls mitotic spindle orientation through affecting the cortical dynein. Most importantly, authors now include statistical evaluation of the main phenotypes after depletion of Anx2 or its interacting partner Ahnak and they conformed those phenotypes in an independent cell line. Further, they provide new data showing that depletion of Ahnak impairs localization of not only dynein and dynactin but also NuMa at the cell cortex.

Comments for the author

Overall, authors successfully addressed all my points during revision and I believe that the manuscript is now suitable for publication.

Reviewer 2
Advance summary and potential significance to field

In this manuscript, Gallaud et al. investigate a role of the Annexin A2(Anx2)/Ahnak complex in mitosis. In previous work, the senior author has demonstrated a role for Anx2 in the Rho-dependent formation of the cleavage furrow during cytokinesis (Benaud et al., Embo Rep 2015). In the current manuscript the authors demonstrate Anx2 is also important during earlier phases of mitosis. Depletion of Anx2 in Hela cells results in mitotic defects, as this strongly delays progression into anaphase, and disrupts spindle orientation that has previously been shown to be instructed by cell-ECM adhesion cues in Hela cells. The authors show Anx2 is necessary to recruit Ahnak to the cell cortex facing spindle poles. Furthermore, they propose the Anx2/Ahnak complex controls spindle orientation through an association with the dynein-dynactin complex which is absent from the cortex near the spindle poles upon Anx2 or Ahnak depletion. Finally, the authors show Ahnak and Anx2 are required the proper cortical localization of NuMA in mitotic cells.

This manuscript adds to the existing knowledge on spindle orientation induced by cell-matrix adhesion cues, as it demonstrates an essential role for the Anx2/Ahnak complex in this process. Furthermore, it demonstrates a role for this complex in anaphase progression. Despite showing a role of Anx2/Ahnak in both of these processes, how exactly these proteins contribute to these processes still remains elusive.

Comments for the author

The authors have included additional experiments and analyses in their revised manuscript to support their conclusions. With these revisions they addressed several of my initial concerns, although some concerns remain. Mechanistic explanations for the observed mitotic phenotypes in Anx2/Ahnak depleted cells and how these phenotypes are connected to each other, still remain a bit elusive. However, the authors now attempt to provide potential explanations in the discussion.
As indicated in my initial comments, the authors needed to validate the observed enrichment of Anx2/Ahnak at the cortex facing spindle poles, for which they now included MyrPalm-GFP controls. However, the authors should not merely show 2 examples of this, but as indicated analyze the cortical distribution of Anx2/Ahank relative to this control protein. This is underscored by the fact that, as indicated in the rebuttal letter, MyrPalm-GFP does not always appear homogenous throughout the cortex (and in Fig 3d even appears somewhat enriched at the cortex near one of the spindle poles). Better analysis of this polarized distribution of Anx2/Ahnak is particularly important as the newly added data shows Anx2/Ahnak are already cortically enriched in interphase, and are not necessarily more recruited to the cortex in mitosis but instead may redistribute along the cortex. Finally, MyrPalm-GFP should also be shown for cells on the L-shaped patterns.
The authors claim that Anx2/Ahnak also become enriched at the cortex facing spindle poles in MDCK cells (Fig. S3A). However, this figure only shows an x-z projection of MDCK cells in which only this part of the cortex is shown. An xy-projection should be included to show that Anx2/Ahnak are enriched at this part of the cortex.

Second revision

Author response to reviewers' comments

Response to Reviews :

Reviewer1 had no further requests

Reviewer2 :
1) As indicated in my initial comments, the authors needed to validate the observed enrichment of Anx2/Ahnak at the cortex facing spindle poles, for which they now included MyrPalm-GFP controls. However, the authors should not merely show 2 examples of this, but as indicated analyze the cortical distribution of Anx2/Ahank relative to this control protein. This is underscored by the

fact that, as indicated in the rebuttal letter, MyrPalm-GFP does not always appear homogenous throughout the cortex (and in Fig 3d even appears somewhat enriched at the cortex near one of the spindle poles). Better analysis of this polarized distribution of Anx2/Ahnak is particularly important as the newly added data shows Anx2/Ahnak are already cortically enriched in interphase, and are not necessarily more recruited to the cortex in mitosis but instead may redistribute along the cortex. Finally, MyrPalm-GFP should also be shown for cells on the L-shaped patterns.

Various studies have shown that the lipid composition of the prometaphase cell plasma membrane is not homogenous. The use of MyrPalm-GFP is therefore not the most accurate tool for signal standardization. For this reason, we have quantified and compared the ratio of protein intensity (cortex/cytoplasm) at distinct specific cortical regions. In patterned cells, we have analyzed protein intensity at the cortex facing the adherent and non-adherent side (Fig. 2J and Fig. 3H). In non-patterned cells, we have compared Anx2 intensity at the cortex facing spindle pole and the equatorial plate (Fig 2 H). For Ahnak, we had previously provided quantification at the polar cortex. However, quantification of cortical Ahnak at the equator was missing. We have now performed this quantification and replaced Fig. 3F with a new panel indicating the quantification of Ahnak intensity at both the spindle pole and the equatorial cortex, and modified accordingly the manuscript p6.

2) The authors claim that Anx2/Ahnak also become enriched at the cortex facing spindle poles in MDCK cells (Fig. S3A). However, this figure only shows an x-z projection of MDCK cells in which only this part of the cortex is shown. An xy-projection should be included to show that Anx2/Ahnak are enriched at this part of the cortex.

Experiments illustrated in Fig.S3 were performed to comply with the request of reviewer 1 to confirm the knockdown phenotype in a less transformed cell line. We used MDCK epithelial cells, which have been widely used to study planar cell division. It has been well documented that in these cells the NuMA/dynein anchoring complex is localized to the lateral domain and excluded from the apical domain (Hao *et al*. Curr Biol 2010 ; Banon- Rodriguez *et al*. EMBO J. 2014 ; Tuncay *et al*. Nature Com. 2015). This localization enables the attachment of astral microtubules to the lateral domain and positioning the division axis parallel to the adhesion surface. The z axial distribution shown in Fig. S3A illustrates that Ahnak is enriched at the lateral domain facing the spindle poles, and is not detected at the apical and basal domain of the cells. We have now clarified this point in the manuscript p7/8. To avoid any confusion with the description and quantification performed in HeLa cells, Fig.2G and H, we have renamed in Fig.2 the cortex facing the metaphase plate equatorial cortex.

Third decision letter

MS ID#: JOCES/2021/259344

MS TITLE: Annexin A2 and Ahnak control cortical NuMA-dynein localization and mitotic spindle orientation

AUTHORS: Aude Pascal, Emmanuel Gallaud, Regis Giet, and Christelle Benaud
ARTICLE TYPE: Research Article

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