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The Drosophila mitotic spindle orientation machinery requires activation, not just localization

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Review #1

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

The manuscript by Neville et al addresses the link between the localization and the activity of the so-called "Pins complex" or "LGN complex", that has been shown to regulate mitotic spindle orientation in most animal cell types and tissues. In most cell types, the polarized localization of the complex in the mitotic cell (which can vary between apical and basolateral, depending on the context) localizes pulling forces to dictate the orientation
The authors reexplore the notion that this polarized localization of the complex is sufficient to dictate spindle orientation, and propose that an additional step of "activation" of the complex is necessary to refine positioning of the spindle.

The experiments are performed in the follicular epithelium (FE), an epithelial sheet of cell that surrounds the drosophila developing oocyte and nurse cells in the ovarium. Like in many other epithelia, cell divisions in the FE are planar (the cell divides in the plane of the epithelium). The authors first confirm that planar divisions in this epithelium depends on the function of Pins and its partner mud, and that the interaction between the two partners is necessary, like in many other epithelial structures. Planar divisions are often associated with a lateral/basolateral "ring" of the Pins complex during mitosis. The authors show that in the FE, Pins is essentially apical in interphase and becomes enriched at the lateral cortex during mitosis, however a significant apical component remains, whereas mud is almost entirely absent from the apical cortex. Pins being "upstream" of mud in the complex, this is a first hint that the localization of Pins is not sufficient to dictate the localization of mud and of the pulling forces.
The authors then replace wt Pins, whose cortical anchoring strongly relies on its interaction with Gai subunits, with a constitutively membrane anchored version (via a N-terminal myristylation). They show that the localization of myr-Pins mimics that of wt-Pins, with a lateral enrichment in mitosis, and a significant apical component. Since a Myr-RFP alone shows a similar distribution, they conclude that the restricted localization of Pins in mitosis is a consequence of general membrane characteristics in mitosis, rather than the result of a dedicated mechanism of Pins subcellular restriction. Remarkably, Myr-Pins also rescues Pins loss-of-function spindle orientation defects.
They further show that the cortical localization of Pins does not require its interaction with Dlg (unlike what has been suggested in other epithelia). However, spindle orientation requires Dlg, and in particular it requires the direct Dlg/Pins interaction. The activity of Dlg in the FE appears to be independent from khc73 and Gukholder, two of its partners involved in its activity in microtubule capture and spindle orientation in other cell types.
Based on all these observations, the authors propose that Dlg serves as an activator that controls Pins activity in a subregion of its localization domain (in this case, the lateral cortex of the mitotic FE cell).
They propose to test this idea by relocalizing Pins at the apical cortex, using Inscuteable ectopic expression. With the tools that they use to drive Inscuteable expression, they obtain two populations of cells. One population has a stronger apical that basolateral

Insc distribution, and the spindle is reoriented along the apical-basal axis; the other population has higher basolateral than apical levels of Insc distribution, and the spindle remains planar. The authors write that Pins localization is unchanged between the two subsets of cells (although I do not entirely agree with them on that point, see below), and that although Mud is modestly recruited to the apical cortex in the first population, it remains essentially basolateral in both. In this situation, the localization of Insc in the cell is therefore a better predictor of spindle orientation than that of Pins or Mud. Remarkably, removing Dlg in an Insc overexpression context leads to a dramatic shift towards apical-basal reorientation of the spindle, suggesting that loss of Dlg-dependent activation of the lateral Pins complex reveals an Insc-dependent apical activation of the complex.

Overall, I find the demonstration convincing and the conclusion appropriate. One of the limitations of the study is the use of different drivers and reporters for the localization of Pins, which makes it hard to compare different situations, but not to the point that it would jeopardize the main conclusions. I do not have major remarks on the paper, only a few minor observations and suggestion of simple experiments that would complete the study

Minor:

What happens to Pins and Mud in Dlg mutant cells that overexpress Insc and behave as InscA? Are they still essentially lateral, or are they more efficiently recruited to the apical cortex?

Regarding the competition between Pins and Insc for dictating the apical versus basolateral localization of Insc, the Insc-expression threshold model could be easily tested in Pins62/62 mutants, where it is expected that only InscA localization should be observed, even at 25{degree sign}C (unless Pins is required for the cortical recruitment of Insc, as it is the case in NBs - see Yu et al 2000 for example)

I do not agree with the authors on P.10 and Figure 6A-D, when they claim that the apical enrichment of Pins is equivalent in both InscA and InscB cells. The number of measured cells is very low, and the ratio of apical/lateral Pins differs between the two sets of cells. The number of cells should be increased and the ratios compared with a relevant statistic method.

A lot of the claims on Pins localization rely on overexpression (generally in a Pins null background) of tagged Pins expressed from different promoters or drivers, and fused to different fluorescent tags. Therefore, it is difficult to evaluate to which extent the localization reflects an endogenous expression level, and to compare the different situations. As the cortical localization of Pins relies on interaction with cortical partners (mostly GDP-bound Gai) which are themselves in limiting quantity in the cell, and in the case of Gai-GDP, regulated by Pins GDI activity, this poses a problem when comparing their distribution, because the expression level of Pins may contribute to its cortical/cytoplasmic ratio, but also to its lateral/apical distribution. Although I understand that the authors have been using tools that were already available for this study, I think it would be more convincing if all the Pins localization studies were

performed with endogenously tagged Pins, even those with Myr localization sequences. In an age of CRISPR-Cas-dependent homologous recombination, I think the generation of such alleles should have been possible. Although this would probably not change the main claims of the paper, it would have made a more convincing case for the localization studies.

The authors should indicate in the figure legends or in the methods that the spindle orientation measurements for controls or Pins62/62 are reused between figures 1, 3, 4, 5, 6 , and between figure 3, 4 and 5, respectively

2. Significance:

Significance (Required)

Altogether, this study makes a convincing case that the localization of the core members of the pulling force complex, Pins and Mud, is not entirely sufficient to localize active force generation, and that the complex must be activated locally, at least in the FE.

The notion of activation of the Pins/LGN complex has probably been in many people's mind for year: Pins/LGN works as a closed/open switch depending on the number of Gai subunits it interacts with, it must be phosphorylated, etc... suggesting that not all cortical Pins/LGN was active and involved in force generation. However the study presented here shows an interesting case where localization and activation are clearly disconnected.
The authors show how Dlg plays this role in physiological conditions in the FE, and use ectopic expression of Insc to show that, at least in an artificial context, Insc can have the same "activating activity" (or at least an activating activity that is stronger than its apical recruitment capability and stronger than Dlg's activating activity). It is to my knowledge the first case of such a clear dissociation.
In their discussion, the authors are careful not to generalize the observation to other tissues. Although I did not reexplore all that has been published on the Pins/LGN-NuMA/Mud complex over the last 20 years, my understanding is that despite interesting cases of distribution of the complex like that of Mud in the tricellular junction in the notum, the localization model can still explain most of the phenotypes that have been described without invoking an activation step. If it is the case, then the activation model is another variation (an interesting one!) on the regulation of the core machinery, which are plentiful as the authors indicate in their introduction, and is maybe specific to the FE; if not, then it would be interesting to push the discussion further by reexamining previous results in other systems, and pinpointing those phenotypes that could be better explained with an activation step.

Overall, I find this is an elegant piece of work, which should be of interest to many cell and developmental biologists beyond the community of spindle orientation aficionados.

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 1 and 3 months

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Reviewer Publons

Yes

Review #2

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

Summary:

The manuscript by Neville et al. addressed the mechanism how conserved spindle regulators (Pins/Mud/Gai/Dynein) control spindle orientation in the proliferating epithelia by revising "the canonical model", using the Drosophila follicular epithelium (FE). The authors examined the epistatic relationship among Pins, Mud and Dlg in FE and found that Pins controls the cortical localization of Mud by utilizing mutant analyses, and suggested their localization does not fully overlap using the newly generated knock-in allele. They also showed that Pins relocalization during mitosis depends on cortical remodeling, or passive model, where Pins localization changes with other membrane-anchored proteins. Their data further suggest that Pins cortical localization is not influenced by Dlg, but Pins-interacting domain of Dlg does affect spindle orientation. Based on these results, the authors propose that Dlg controls spindle orientation not by redistributing Pins, but by promoting (or "activating" from their definition) Pins-dependent spindle orientation. Interestingly, ectopic expression of Inscuteable (Insc) suggested that Insc localization, either apical or lateral, correlates with spindle orientation, and their localization is a dominant indicator of spindle orientation, compared to the localization of Pins and Mud, implicating potentially distinct roles of activation and localization of the spindle complex. Overall their genetic experiments are well-designed and provide stimulation for future research. However,

their evidence is suggestive, but not conclusive for their proposal. I have several concerns about their conclusion and would like to request more detailed information as well as to propose additional experiments.

Major concerns:

1. This report lacks technical and experimental details. As the typical fly paper, the authors need to show the exact genotypes of flies they used for experiments. This needs to be addressed for Figures 1-6, and Supplemental Figures. Especially, which Gal4 drivers were used for UAS-Pins wt or mutant constructs in Figure 4 with pins mutant background, Khc73, GUKH mutant backgrounds. Which exact flies were used for mutant clone experiments for Supplemental Figure 3? (A for typical mosaic, and B for MARCM). Without these details, it is impossible to evaluate results and reproduce by others.
2. Related to the comment 1, how did the author perform "clonal expression of Ubi-Pin-YFP" in page 5? As far as I understand, Ubi-Pin-YFP is expressed ubiquitously by the ubiquitin promoter.
3. In page 6, if Pins relocalization is passive and is associated with membrane-anchored protein remodeling during mitosis, its relocalization can be suppressed by disrupting the process of mitotic remodeling (mitotic rounding). The authors should test this by either genetic disruption or pharmacological treatment for the actomyosin should cause defects in Pins relocalization, which bolster their conclusion.
4. The critical message in this manuscript is that the core spindle complex mediated by Pins-Mud controls spindle orientation by "activation", but not localization. The findings that Pins and Mud localization is not influenced by Insc and that ecotpic Insc expression and genetic Mud depletion (Figure 6) might support their proposal, but these results just suggest their localization does not matter. I wonder how the authors could conclude and define "activation". What does this activation mean in the context of spindle orientation? Can the authors test activation by enzymatic activity or assess dynamics of spindle alignment?
5. In page 7-8, although Pins-S436D rescue spindle orientation, but Pins-S436A does not in pins null clones background, Pins localization is not influenced by Dlg. This questions how exactly Pins and Dlg can interact, and how Dlg affect Pins function. Related to this observation, in the embryonic Pins:Tom localization in dlg mutant does not provide strong evidence to support their conclusion given the experimental context is different from previous study (Chanet et al., 2017).
6. In page 11, the authors state "... that activation of pulling in the FE requires Dlg". I was not convinced by anything related to "pulling". There is no evidence to support "pulling" or such dynamic in this paper, just showing Mud localization, correct?
7. Ectopic expression of Insc (Figure 6) provided a new idea and hypothesis, but the conclusion is more complicated given that Insc is not expressed in normal FE. For example, the statement that "Inscuteable and Dlg mediate distinct and competitive mechanism for activation of the spindle-orienting machinery in follicle cells" is probably right, but it does not show anything meaningful since Insc does not exist in normal FE. Is Dlg in a competitive situation during mitosis of FE? If so, which molecules are competitive against Dlg? The important issue is to provide a new interpretation of how spindle orientation is controlled epithelial cells. I strongly recommend to add models in this manuscript for clarity.

Minor comments:

8. Some sections were not written well in the manuscript. "It does not" in page 6. "These predictions are not met". I just couldn't understand what they stand for. Their writing has to be improved.
9. In page 9, Supplementary Figure 4 should be cited in the paragraph (A potential strategy for..), not Supplemental Figure 1A, and 1B.
10. In page 10, the authors examine aPKC localization in Insc expressing context of FE. Does aPKC localization correlate with Insc localization (Insc dictates aPKC?)? aPKC is not involved in spindle orientation from the author's report (Bergstralh et al., 2013), so it does not likely provide any supportive evidence.
11. In Dicussion page 12, "In addition, we find that while the LGN S408D (Drosophila S436D) variant is reported to act as a phosphomimetic, expression of this variant has no obvious effect on division orientation (Johnston et al., 2012)". Where is the evidence for this? I interpret that this phosphomimetic form can rescue like wt-Pins not like unphospholatable mutant S436A, so it means that have an effect on spindle orientation, correct?

2. Significance:

Significance (Required)

The authors showed that Pins and Mud localization themselves are not sufficient for the control of spindle orientation with genetic analyses. While the authors tried to challenge the concept of "canonical model", there is no clear demonstration of "activation" of the spindle complex. I appreciate their genetic evidence and new results, and understand the message that Pins and Mud effects are beyond localization, but there is no alternative mechanism to support their model. At the current stage, their evidence provides more hypothesis, not conclusion. Based on my expertise in Developmental and Cell biology, I suggest that the work has an interest in audience who studies spindle machinery, but for general audience.

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 1 and 3 months

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Reviewer Publons

Yes

Review #3

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

Neville et al re-examine the role and regulation of Pins/LGN in Drosophila follicular epithelial cells. They argue that polar or bipolar enrichment of Pins localisation at the plasma membrane is not crucial for spindle orientation, and therefore propose that Pins must be somehow activated to function. These interpretations are not supported by the data. However, the data strongly suggest an alternative interpretation which is of major biological significance.

Comments:

1. In the experiments on Dlg mutants (Fig 4D, S3) visualising Pins:Tom, the wild-type needs to be shown next to the Dlg mutant image, otherwise a comparison cannot be made. For example, Pins:Tom looks strongly enriched at the lateral membranes in the wild-type shown in Fig 2B&C, but much more weakly localised at the lateral membranes in Dlg1P20/2 mutants in Fig 4D. Thus, it looks like the Dlg GUK domain is required for full enrichment of Pins:Tom at lateral membranes, even if some low level of Pins can still bind to the plasma membrane in the absence of the Dlg GUK domain. Quantification would likely show a reduction in Pins:Tom lateral enrichment in the Dlg1P20/2 mutants - consistent with the spindle misorientation phenotype in these mutants.
2. In Fig 4E, the phosphomutant PinsS436A-GFP looks more strongly apical and less strongly lateral than the wildtype Pins-GFP, consistent with the spindle misorientation phenotype in S436A rescued pins mutants.
3. In the InscA examples, Pins:Tom looks apical. In the InscB examples, Pins:Tom looks more laterally localised, consistent with the spindle orientations in these experiments.

Thus, these data appear to support the existing model that Pins enrichment at the plasma membrane is a key factor directing mitotic spindle orientation in these cells. The author's claim in the final sentence of the abstract "Local enrichment of Pins is not sufficient to determine spindle orientation; an activation step is also necessary" is not supported by the data.

The open question posed by the data is why GFP-Mud is excluded apically & basally during mitosis, while Pins:Tom is not. The simple alternative model is that Mud only localises to the plasma membrane where Pins is most strongly concentrated, such that Mud strongly amplifies any Pins asymmetry. Thus, even myr-Pins can still rescue a pins

mutant, because myr-Pins is still enriched laterally compared to apically (or basally).

Thus, I would strongly suggest re-titling the manuscript to: "Mud/NuMA amplifies minor asymmetries in Pins localisation to orient the mitotic spindle".

Mud/NuMA presumably achieves this amplification by binding to the plasma membrane only where Pins is concentrated above a critical threshold level. This would mean a non-linear model based on cooperativity among Pins monomers that increases the binding avidity to Mud above the threshold concentration of Pins monomers.

2. Significance:

Significance (Required)

The manuscript is focused on the question of mitotic spindle orientation in epithelial cells, which is a fundamental unsolved problem in biology. The data reported are impressive and important, providing new insights into how the key spindle orientation factors Mud/NuMA and Pins/LGN localise during mitosis in epithelia. I recommend publication after major revisions.

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Less than 1 month

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Reviewer Publons

Yes

1. General Statements [optional]

We got a sort of Goldilocks set of reviews.

Reviewer 1 was exceedingly supportive, and gave some terrific, useful feedback. They concluded that our study will be of interest to a wide audience of cell and developmental biologists. We happily agree, and are therefore sending it to EMBO J, where we've had a great experience in the past.

Reviewer 2 was a bit more negative, though this seems to be a matter of philosophy if not (in some cases) semantics. Our impression is that their major comments were fairly minor, and that their evaluation of the work is not too far off from our own.

Reviewer 3 finds our data is "impressive and important," but has asked us to reinterpret it according to a different model, namely that Pins localization is sufficient to orient the spindle. This model is fundamentally and forcefully contradicted by our work. We have endeavored to clarify that message during the initial revision.

2. Description of the planned revisions

Reviewer 1's comments:

Regarding the competition between Pins and Insc for dictating the apical versus basolateral localization of Insc, the Insc-expression threshold model could be easily tested in Pins62/62 mutants, where it is expected that only InscA localization should be observed, even at 25{degree sign}C (unless Pins is required for the cortical recruitment of Insc, as it is the case in NBs - see Yu et al 2000 for example).

This is another great experiment and one we'd love to carry out. Again, the genetics are currently challenging, only because both UAS-Inscuteable and FRT82B *pins*^{p62} are on the third chromosome. (Right now we're trying to hop UAS-Inscuteable to the second).

However, we do have another idea for testing the threshold model, which is to repeat the experiment in which we express UAS-Insc in cells that are *Dlg*$^{IP20/IP20}$ at 25°C. Because the relevant cells (UAS-Insc OX in *Dlg* mitotic clones) are relatively rare, we have not yet been able to collect enough examples to make a firm conclusion. However, our preliminary results (only six cells so far!) suggest that more Insc^{B} cells are observed at the lower temperature, consistent with the threshold model.

Revision Plan

I do not agree with the authors on P.10 and Figure 6A-D, when they claim that the apical enrichment of Pins is equivalent in both InscA and InscB cells. The number of measured cells is very low, and the ratio of apical/lateral Pins differs between the two sets of cells. The number of cells should be increased and the ratios compared with a relevant statistic method.

Totally fair. We are working to add more data to these panels (6B and 6D). The trend observed in 6D may be softening in agreement with the reviewer's prediction, although we currently don't yet have enough new data points to be confident in that conclusion. Therefore, we have not yet updated the manuscript, though we expect to do so during the revision period. We will also add a statistical comparison. Importantly, as the reviewer suggested, this does not alter our conclusions.

Reviewer 2's comments:

5. In page 7-8, although Pins-S436D rescue spindle orientation, but Pins-S436A does not in pins null clones background, Pins localization is not influenced by Dlg. This questions how exactly Pins and Dlg can interact, and how Dlg affect Pins function. Related to this observation, in the embryonic Pins:Tom localization in dlg mutant does not provide strong evidence to support their conclusion given the experimental context is different from previous study (Chanet et al., 2017).

We agree with the reviewer. Our data (this paper and previous papers) and the work of others indicate that this interaction is important for spindle orientation (Bergstralh et al., 2013a; Saadaoui et al., 2014; Chanet et al., 2017). However, we show here that Dlg doesn't obviously impact Pins localization (as proposed in our earlier paper), but does impact the ability of the spindle orientation machinery to work (hence activity).

The reviewer makes a very good point. Our experimental context is different from the previous study concerning Pins and Dlg in embryos: Chanet *et al* (2017) performed their work in the embryonic head, whereas we look at divisions in the ventral embryonic ectoderm (Figure 2A). These are distinct mitotic zones (Foe *et al.* (1989) Development) and exhibit distinct epithelial morphologies. We show that Pins:Tom localizes at the mitotic cell cortex in *Dlg[2]/Dlg[1P20]* in cells in the ventral embryonic ectoderm. Our only conclusion from this experiment is that Pins:Tom can localize without the Dlg GUK domain in another cell type (outside the follicular epithelium). In the current preliminary revision we have softened our claim as follows:

"We also examined the relationship between Pins and Dlg in the *Drosophila* embryo. A previous study showed that cortical localization of Pins in embryonic head epithelial cells is lost when *Dlg* mRNA is knocked down (Chanet et al., 2017). We find that Pins:Tom localizes to the cortex in the ventral ectoderm of early embryos from Dlg^{1P20}/Dlg^{2} mothers, indicating that Pins localization in the ventral embryonic ectoderm epithelium does not require direct interaction with Dlg. We therefore speculate that Dlg plays an additional role in that tissue, upstream of Pins (Figure 4G)."

Our intention is to elaborate on our findings with additional data from embryos. To this end we have already acquired preliminary control data (Figure 2) investigating the spindle angle with respect to the plane of the epithelium, and are in the process of examining spindle angles in *dlg* mutant embryonic tissue.

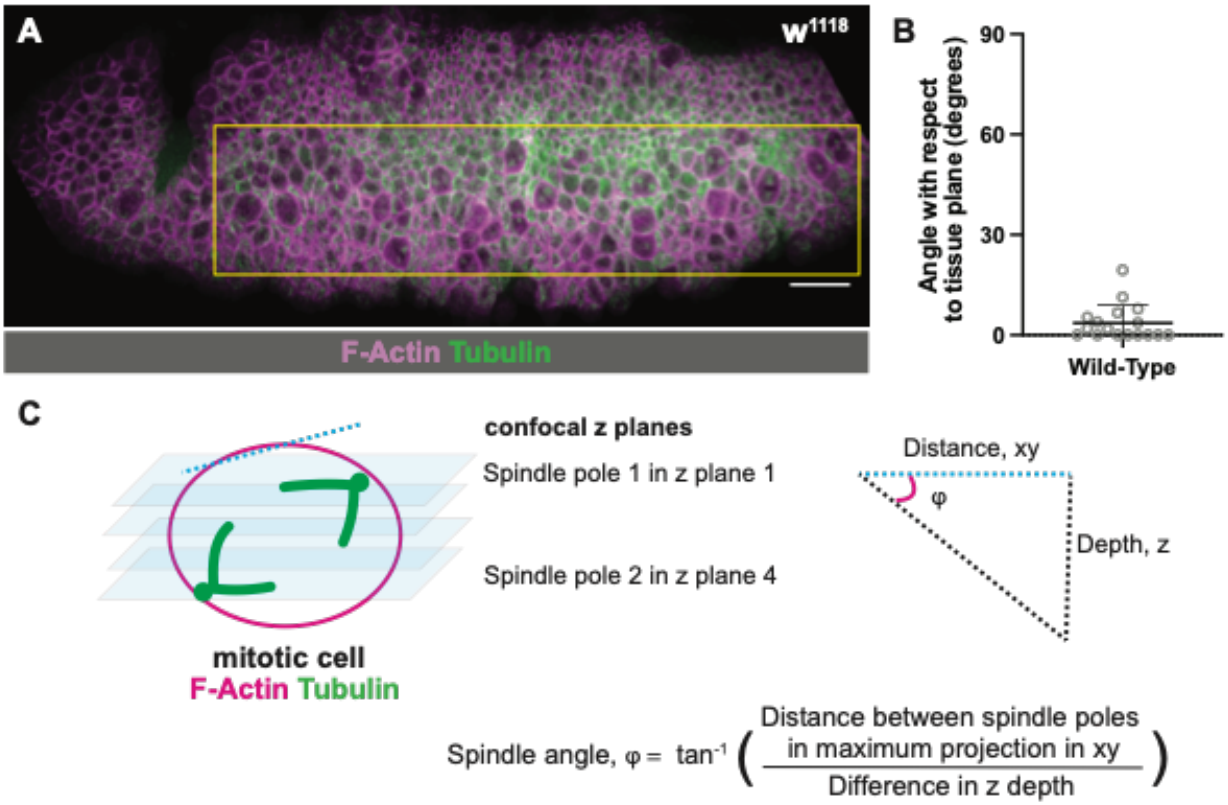

Figure 1 – Examination of spindle angles in the embryonic ectoderm. A) Lateral view of a fixed and immunostained Stage 10 embryo positioned anterior (left) imaged on using a confocal microscope. Image is a maximum-intensity projection of 20 z planes. We are focusing on the divisions that occur in the ventral ectoderm domain highlighted by the yellow box. Mitotic cells can be clearly identified by their cell shape and Tubulin cytoskeleton morphology. B) Quantification of spindle angles of mitotic cells from the ventral embryonic ectoderm in w1118 embryos. N = 17 cells across 2 embryos. C) Description of the method used to obtain spindle angles in the embryonic ectoderm. Only cells from tissue that has been flattened against the coverslip are used for quantification.

3. Description of the revisions that have already been incorporated in the transferred manuscript

Reviewer 1's comments:

A lot of the claims on Pins localization rely on overexpression (generally in a Pins null background) of tagged Pins expressed from different promoters or drivers, and fused to different fluorescent tags. Therefore, it is difficult to evaluate to which extent the localization reflects an

endogenous expression level, and to compare the different situations. As the cortical localization of Pins relies on interaction with cortical partners (mostly GDP-bound Gai) which are themselves in limiting quantity in the cell, and in the case of Gai-GDP, regulated by Pins GDI activity, this poses a problem when comparing their distribution, because the expression level of Pins may contribute to its cortical/cytoplasmic ratio, but also to its lateral/apical distribution. Although I understand that the authors have been using tools that were already available for this study, I think it would be more convincing if all the Pins localization studies were performed with endogenously tagged Pins, even those with Myr localization sequences. In an age of CRISPR-Cas-dependent homologous recombination, I think the generation of such alleles should have been possible. Although this would probably not change the main claims of the paper, it would have made a more convincing case for the localization studies.

We don't disagree at all with this point. We did indeed try to stick with the published UAS-Pins-myr-GFP, not only for convenience but because it allows us to make comparisons to other studies using the same tool (Chanet *et al* Current Biology 2017 and Camuglia *et al* eLife 2022). Another consideration is that we used only one driver across our experiments (Traffic jam-GAL4). It is quite weak at the developmental stages that we examine, meaning that overexpression is not a major concern. (Indeed we have struggled with the opposite problem).

We certainly take the reviewer's comment seriously and we therefore described it in the manuscript. We are currently working to develop endogenous tools using CRISPR.

Paragraph added to Discussion – Limitations of our Study:

"Another technical consideration is that our work makes use of transgenes under the control of Traffic jam-GAL4. While this strategy allows us to compare our results with previous work employing the same or similar tools, a drawback is that we cannot guarantee that Traffic jam-GAL4 drives equivalent expression to the endogenous Pins promoter (Chanet et al., 2017, Camuglia et al., 2022). However, given that Traffic jam-GAL4 is fairly weak at the developmental stages examined, we are not especially concerned about overexpression effects."

The authors should indicate in the figure legends or in the methods that the spindle orientation measurements for controls for Pins62/62 are reused between figures 1, 3, 4, 5, 6 , and between figure 3, 4 and 5, respectively.

Absolutely. Added to the Methods section.

Reviewer 2's comments:

Major concerns:

1. This report lacks technical and experimental details. As the typical fly paper, the authors need to show the exact genotypes of flies they used for experiments. This needs to be addressed for Figures 1-6, and Supplemental Figures. Especially, which Gal4 drivers were used for UAS-Pins wt or mutant constructs in Figure 4 with pins mutant background, Khc73, GUKH mutant

backgrounds. Which exact flies were used for mutant clone experiments for Supplemental Figure 3? (A for typical mosaic, and B for MARCM). Without these details, it is impossible to evaluate results and reproduce by others.

We take this concern very seriously!

- We listed the GAL4 driver (Traffic jam-GAL4) in the first section of the Materials and Methods: *Expression was driven by Traffic Jam-GAL4 (Olivieri et al., 2010).* The transgene and relevant citation have been added to Table 1.
- We explained the background stock for the MARCM experiment in the Materials and Methods: *Mosaic Analysis with a Repressible Cell Marker (after the method of Lee and Luo) was carried out using GFP-mCD8 (under control of an actin promoter) as the marker.* The transgene and relevant citation have been added to Table 1.
- In line with other fly studies (eg. Nakajima *et al.,* Nature 2013) and our own *Drosophila* work (Bergstralh *et al* Current Biology 2013, Bergstralh*, Lovegrove*, St Johnston NCB 2015, Bergstralh *et al* Development 2016, Finegan *et al* EMBO J 2019, Cammarota*, Finegan* *et al* Current Biology 2020) we were careful to show the relevant genotype components in each figure.
- We included a fully referenced Supplementary Table (Table 1 – *Drosophila* genetics) listing every mutant allele or transgene with a citation and a note about availability. We have expanded this table in response to the author's concern (see above).

2. Related to the comment 1, how did the author perform "clonal expression of Ubi-Pin-YFP" in page 5? As far as I understand, Ubi-Pin-YFP is expressed ubiquitously by the ubiquitin promoter.

The reviewer makes a good point. We regret that we did not make this experiment more clear. Ubi-Pins-YFP was recombined onto an FRT chromosome (FRT82B). We made mitotic clones.

We have clarified this in the Methods section as follows:

"Mitotic clones of Ubi-Pins-YFP were made by recombining the Ubi-Pins-YFP transgene onto the FRT82B chromosome"

4. The critical message in this manuscript is that the core spindle complex mediated by Pins-Mud controls spindle orientation by "activation", but not localization. The findings that Pins and Mud localization is not influenced by Insc and that ecotpic Insc expression and genetic Mud depletion (Figure 6) might support their proposal, but these results just suggest their localization does not matter. I wonder how the authors could conclude and define "activation". What does this activation mean in the context of spindle orientation? Can the authors test activation by enzymatic activity or assess dynamics of spindle alignment?

We intend for the critical message of the manuscript to be that "The spindle orienting machinery requires activation, not just localization." We absolutely do not make the claim that localization is

not important, only that it is not sufficient. The reviewer recognizes this point here and in a subsequent comment: "The authors showed that Pins and Mud localization themselves are not sufficient for the control of spindle orientation with genetic analyses."

We also do not claim that Pins and/or Mud localization are not impacted by Inscuteable. On the contrary, we plainly see and report that they are; the intensity profiles in Figure 6 are distinct from those in Figure 2, as discussed in the text.

We appreciate the reviewer's point about activation. Since we do not understand these proteins to be enzymes, we aren't sure what enzymatic activity would be tested. The dynamics of spindle alignment in this slowly developing system are prohibitively difficult to measure: the mitotic index is very low (~2%) and only a very small fraction of those cells will be in a focal plane that permits accurate live imaging in the apical-basal axis. Alternative modes of activation include conformational change and/or a connection with other important molecules. The simplest possibility would be that Dlg allows Pins to bind Mud, but so far our data do not support it. We have added the following paragraph to our discussion:

"The mechanism of activation remains unclear. While the most straightforward possibility is that Dlg promotes interaction between Pins and Mud, our results show that Mud is recruited to the cortex even when *Dlg* is disrupted (Figure 4D). Alternatively, Discs large may promote a conformational change in the spindle-orientation complex and/or a change in complex composition. Furthermore, the Inscuteable mechanism is not likely to work in the same way. Dlg binds to a conserved phosphosite in the central linker domain of Pins and should therefore allow for Pins to simultaneously interact with Mud (Johnston et al., 2009). Contrastingly, binding between Pins and Inscuteable is mediated by the TPR domains of Pins, meaning that Mud is excluded (Culurgioni et al., 2011; 2018). While a stable Pins-Inscuteable complex has been suggested to promote localization of a separate Pins-Mud-dynein complex, our work raises the possibility that it might also or instead promote activation."

6. In page 11, the authors state "... that activation of pulling in the FE requires Dlg". I was not convinced by anything related to "pulling". There is no evidence to support "pulling" or such dynamic in this paper, just showing Mud localization, correct?

We appreciate the reviewer's concern. The original sentence read that "*We interpret [our data] to mean that interaction between Pins and Dlg, which is required for pulling, stabilizes the lateral pulling machinery even if Dlg is not a direct anchor*." This statement is based on work across multiple systems, including the *C. elegans* embryo (Grill *et al* Nature 2001), the *Drosophila* pupal notum (Bosveld *et al,* Nature 2016), and HeLa cells (Okumura *et al eLife* 2018), which shows that Mud/dynein-mediated pulling (on astral microtubules) orients/positions spindles. This is described in the introduction.

To address the reviewer's particular concern, we have replaced "pulling" with "spindle-orentation machinery," so that this sentence now reads …"activation of the spindle-orientation machinery in the FE requires Dlg."

9. In page 9, Supplementary Figure 4 should be cited in the paragraph (A potential strategy for..), not Supplemental Figure 1A, and 1B.

Good catch, thank you! We have corrected this.

11. In Dicussion page 12, "In addition, we find that while the LGN S408D (Drosophila S436D) variant is reported to act as a phosphomimetic, expression of this variant has no obvious effect on division orientation (Johnston et al., 2012)". Where is the evidence for this? I interpret that this phosphomimetic form can rescue like wt-Pins not like unphospholatable mutant S436A, so it means that have an effect on spindle orientation, correct?

The reviewer makes a good point. We regret the confusion. We mean to point out that the S436D variant is no different from the wild type. We have amended the text to clarify:

"In addition, we find that while the LGN S408D (*Drosophila* 436D) variant is reported to act as a phosphomimetic, this variant does not cause an obvious mutant phenotype in the follicular epithelium (Johnston et al., 2012). What then is the purpose of this modification? Since the phosphosite is highly conserved through metazoans, one possibility to consider is that the phosphorylation regulates the spindle orientation role of Pins, whereas unphosphorylated Pins plays a different role (Schiller and Bergstralh, 2021)."

Reviewer 3's comments:

1. In the experiments on Dlg mutants (Fig 4D, S3) visualising Pins:Tom, the wild-type needs to be shown next to the Dlg mutant image, otherwise a comparison cannot be made. For example, Pins:Tom looks strongly enriched at the lateral membranes in the wild-type shown in Fig 2B&C, but much more weakly localised at the lateral membranes in Dlg1P20/2 mutants in Fig 4D. Thus, it looks like the Dlg GUK domain is required for full enrichment of Pins:Tom at lateral membranes, even if some low level of Pins can still bind to the plasma membrane in the absence of the Dlg GUK domain. Quantification would likely show a reduction in Pins:Tom lateral enrichment in the Dlg1P20/2 mutants - consistent with the spindle misorientation phenotype in these mutants.

The reviewer raises a reasonable concern about Figure 4D. We noted the difficulty of imaging Pins:Tom, which is exceedingly faint, in our original manuscript. For technical reasons, only one copy of the transgene was imaged in the experiment presented in 4G (two copies were used in Figure 2B), and the lack of signal presented an even greater challenge. In the manuscript we went with the clearest image. To address the reviewer's concern, we have added signal intensity plots to this figure showing that Pins:Tom and Pins-myr are both laterally enriched at mitosis in *Dlg[1P20]/Dlg[2]* mutants. These data have been added as a new panel (E) in Figure 4. We were also able to replace the pictures in 4D with new ones generated after review.

4. Description of analyses that authors prefer not to carry out

Reviewer 1's comments:

What happens to Pins and Mud in Dlg mutant cells that overexpress Insc and behave as InscA? Are they still essentially lateral, or are they more efficiently recruited to the apical cortex?

This is a terrific question. Of course we would love to know and intend to find out.

One way to do this (consistent with the manuscript) would be to generate flies that are *Dlg[1P20],* FRT19A/RFP-nls, hsflp, FRT19A; TJ-GAL4/+; Pins-Tom, GFP-Mud/UAS-Insc. (Note that these flies would only allow us to image Mud; we would have to repeat the experiment using GFP FRT19A; hsflp 38 to see Pins. This isn't ideal given that we'd like to image both together). Generating these flies is a major technical challenge because of the number of transgenes and chromosomes involved.

Our preferred way to do this would be to generate flies that are *Dlg[1P20]/Dlg[2]*; TJ-GAL4/+; Pins-Tom, GFP-Mud/UAS-Insc. So far, we've been unsuccessful. We are now undertaking a modified crossing scheme that we hope will solve the problem, though we aren't overly optimistic about the outcome. We find that the temperature-sensitive mutation *Dlg[2]* presents an activation barrier; while we are able to generate flies that are *Dlg[2]* / FM7 in combination with transgenes and/or mutations on other chromosomes, we do not always recover the *Dlg[2]* / Y males (which must develop at 18degrees) from these complex genotypes.

In the longer term (outside the scope of revision), we are working to develop more tools for imaging Mud and Pins that we hope will help answer this question.

Reviewer 2's comments:

3. In page 6, if Pins relocalization is passive and is associated with membrane-anchored protein remodeling during mitosis, its relocalization can be suppressed by disrupting the process of mitotic remodeling (mitotic rounding). The authors should test this by either genetic disruption or pharmacological treatment for the actomyosin should cause defects in Pins relocalization, which bolster their conclusion.

We agree that this is a cool experiment and are happy to give it another shot. However, we do note that interpretation could be difficult. We don't know that mitotic rounding and membrane-anchored protein remodeling during mitosis are inextricably linked. Notably, the remodeling we describe reflects cell polarity; apical components are evidently moved to the lateral cortex. This is contrary to understanding of rounding, which reflects isotropic actomyosin activity (Chanet et al., (2017) Curr.Biol. & Rosa et al., (2015) Dev. Cell.). Therefore we don't understand what a "negative" result would mean, or for that matter that a "positive" result would be safe to interpret.

Revision Plan

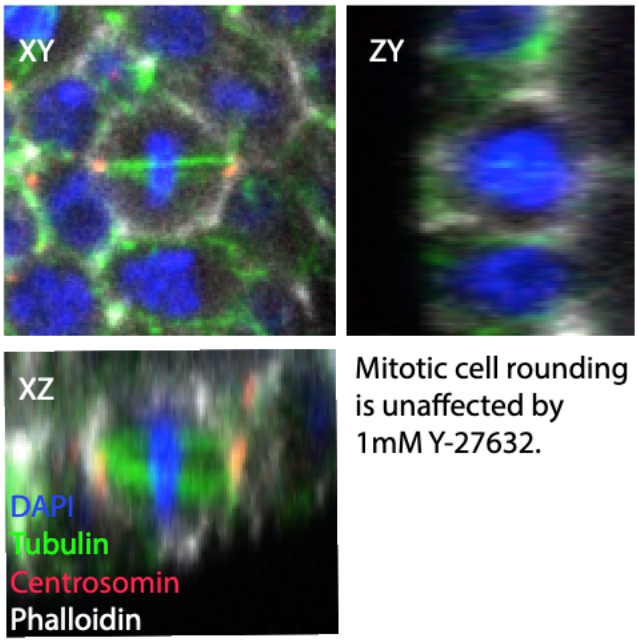

Figure 2- The follicular epithelium shows not phenotypic effect on cell rounding when injected with 1mM Y-27632.

We have attempted many strategies to prevent cell rounding in the follicular epithelium, none of which have successfully prevented rounding. 1) We attempted to genetically knockdown Moesin in the FE and did not see an effect on cell rounding. However we couldn't confirm knockdown and therefore are not confident in this manipulation. 2) It is difficult to interpret the result of genetically disrupting Myosin, because it causes pleiotropic effects, such as inhibition of the cell cycle, and disruption of monolayer architecture. 3) We treated egg chambers with Y-27632 (a Rok-inhibitor) and examined its effect on mitotic cell rounding and on cytokinesis, which are Rok-dependent processes. Our experiments were performed using manually-dissociated ovarioles treated for 45 minutes in Schneider Cell Medium supplemented with insulin. Even at our maximum concentration of 1mM Y-27632, several orders of magnitude above the K_i, we are unable to see any effect on mitotic cell shape or actin accumulation at the mitotic cortex and did not observe any evidence of defective cytokinesis (Figure 1). We also did not observe defects in spindle organization or orientation, as would be expected from failed rounding. We therefore do not believe that the inhibitor works in this tissue. One possible explanation is that the follicle cells are secretory, and likely to pass molecules taken up from the media quickly into the germline. Therefore, we do not anticipate that we can perform this experiment to our satisfaction.

7. Ectopic expression of Insc (Figure 6) provided a new idea and hypothesis, but the conclusion is more complicated given that Insc is not expressed in normal FE. For example, the statement that "Inscuteable and Dlg mediate distinct and competitive mechanism for activation of the spindle-orienting machinery in follicle cells" is probably right, but it does not show anything meaningful since Insc does not exist in normal FE. Is Dlg in a competitive situation during mitosis of FE? If so, which molecules are competitive against Dlg? The important issue is to provide a new interpretation of how spindle orientation is controlled epithelial cells. I strongly recommend to add models in this manuscript for clarity.

We considered the addition of model cartoons very carefully in preparing the original manuscript, and again after review. While we are certainly not going to "dig in" on this point, our concern is that model figures would obscure rather than clarify the message. As the reviewer points out, we do not understand how activation works, and as discussed in the manuscript we don't think it's likely to work the same way in follicle cells (Dlg) as it does in neuroblasts (Insc). Therefore model figure(s) are premature.

Revision Plan

We do not agree with the statement that "Inscuteable and Dlg mediate distinct and competitive mechanism for activation of the spindle-orienting machinery in follicle cells… does not show anything meaningful." This is a remarkable finding because it suggests that there is more than one way to activate Pins. Given the critical importance of spindle orientation in the developing nervous system, and the evolutionary history of the Dlg-Pins interaction, we think that this finding supports a model in which the Dlg-Pins interaction evolved in basal organisms, and a second Inscuteable-Pins interaction evolved subsequently to support neural complexity. These ideas are raised in the Discussion.

The reviewer also writes that "The important issue is to provide a new interpretation of how spindle orientation is controlled epithelial cells." We find this concern perplexing, since the reviewer clearly recognizes that we have provided a new interpretation: Dlg is not a localization factor but rather a licensing factor for Pins-dependent spindle orientation.

Minor comments:
8. Some sections were not written well in the manuscript. "It does not" in page 6. "These predictions are not met". I just couldn't understand what they stand for. Their writing has to be improved.

Again, we are not going to dig in here, but we would prefer to retain the original language, which in our opinion is fairly clear. Our study is hypothesis-driven and based on assumptions made by the current model. We used direct language to help the reviewer understand what happened when we tested those assumptions.

10. In page 10, the authors examine aPKC localization in Insc expressing context of FE. Does aPKC localization correlate with Insc localization (Insc dictates aPKC?)? aPKC is not involved in spindle orientation from the author's report (Bergstralh et al., 2013), so it does not likely provide any supportive evidence.

I'm afraid we don't entirely understand this comment. The interdependent relationship between aPKC and Inscuteable localization is long-established in the literature and was previously addressed in the follicle epithelium (Bergstralh et al. 2016). We do not make the claim here that aPKC governs spindle orientation. We are emphasizing that the difference between InscA and InscB cells extends to the relocalization of polarity components involved in Insc localization. As described in the manuscript, these data are provided to support our threshold model:

"In agreement with interdependence between Inscuteable and the Par complex, we find that aPKC is stabilized at the apical cortex in Insc^{A} cells but enriched at the lateral cortex in Insc^{B} cells (Figure 6E). This finding is consistent with an Inscuteable-expression threshold model; below the threshold, Pins dictates lateral localization of Inscuteable and aPKC. Above the threshold, Inscuteable dictates apical localization of Pins and aPKC."

2. In Fig 4E, the phosphomutant PinsS436A-GFP looks more strongly apical and less strongly lateral than the wildtype Pins-GFP, consistent with the spindle misorientation phenotype in S436A rescued pins mutants.

The reviewer has an eagle eye! We did not detect a difference in localization across the three transgenes, though we were certainly looking for it (that's why we generated these flies in the first place). Again, the strength of signal was a major challenge in these experiments, and we therefore went with the cleanest image. In response to the reviewer's concern, we note that the S436A and S436D examples shown have equivalent apical signal, but only the S436A fails to rescue spindle orientation.

Together, Reviewer Comments 1 and 2 suggest a model in which Dlg is required for lateral enrichment of Pins at mitosis. As described in the manuscript, this is the very model proposed in our own previous study (Bergstralh, Lovegrove, and St Johnston; 2013), and reiterated in a subsequent review article (Bergstralh, Dawney, and St Johnston; 2017). We point these publications out because the senior author of the current manuscript is not especially enthusiastic about showing himself to be wrong (twice!) in the literature. He therefore insisted on seeing multiple lines of evidence before making the counterargument:

1) The reviewer's model (the 2013 model) is firstly challenged by work shown in Figure 3. We find that membrane-anchored proteins (even just myristoylated RFP!) demonstrate lateral enrichment at mitosis, regardless of whether or not they interact with the Dlg-GUK domain.
2) Even stronger evidence is shown in Figure 4F. Pins-myr-GFP is very plainly enriched at the lateral cortex in *Dlg[IP20]/Dlg[2]* mutant cells (now demonstrated with signal intensity plots in FIGURE 4E). However, the spindle doesn't orient correctly (quantified in 4C). Since Dlg is impacting spindle orientation independently of Pins localization, these data support our "claim in the final sentence of the abstract '*Local enrichment of Pins is not sufficient to determine spindle orientation; an activation step is also necessary*'."

3. In the InscA examples, Pins:Tom looks apical. In the InscB examples, Pins:Tom looks more laterally localised, consistent with the spindle orientations in these experiments.

These figures (6A-D) don't only show/quantify Pins:Tom localization. They also show localization of GFP-Mud. Whereas Pins:Tom is certainly apically enriched in the InscA examples, the interesting finding is that GFP-Mud is not. In strong contrast, it instead shows a weak apical localization and a strong lateral enrichment. As described in the manuscript, this pattern of Mud localization predicts normal spindle orientation, which is not observed in these cells.

Thus, these data appear to support the existing model that Pins enrichment at the plasma membrane is a key factor directing mitotic spindle orientation in these cells. The author's claim

in the final sentence of the abstract "Local enrichment of Pins is not sufficient to determine spindle orientation; an activation step is also necessary" is not supported by the data.

We are pleased that the reviewer shared this quote; our claim is that Pins localization is not sufficient, not that it is unnecessary (see above). We absolutely do not dispute that "Pins enrichment at the plasma membrane is a key factor directing mitotic spindle orientation."

The open question posed by the data is why GFP-Mud is excluded apically & basally during mitosis, while Pins:Tom is not. The simple alternative model is that Mud only localises to the plasma membrane where Pins is most strongly concentrated, such that Mud strongly amplifies any Pins asymmetry. Thus, even myr-Pins can still rescue a pins n mutant, because myr-Pins is still enriched laterally compared to apically (or basally).

Thus, I would strongly suggest re-titling the manuscript to: "Mud/NuMA amplifies minor asymmetries in Pins localisation to orient the mitotic spindle".

Well, that is a good-looking title, and we're therefore sorry to decline the suggestion. However, as described above, Figure 4D shows that Pins enrichment does not always predict spindle orientation. More importantly, Figure 6A (cited by the reviewer in Comment 3) very plainly shows that Mud does *not* "only locali[ze] to the plasma membrane where Pins is most strongly concentrated." In this picture – and across multiple InscA cells (Figure 6B) - Pins is strongly concentrated at the apical surface, whereas Mud is not.

Mud/NuMA presumably achieves this amplification by binding to the plasma membrane only where Pins is concentrated above a critical threshold level. This would mean a non-linear model based on cooperativity among Pins monomers that increases the binding avidity to Mud above the threshold concentration of Pins monomers.

This is essentially a minor revision of the standard model, which we expected would hold true in the FE. As described above, it is not supported by our data.

We are delighted that the reviewer finds our data impressive and important, and our experiments insightful. We understand that the "major revisions" requested are meant to bring the paper in line with their model (our own earlier model). Since the data in our original manuscript contradict that model, the revisions are instead focused on clarifying and strengthening our message.

Dear Dr. Bergstralh,

Thank you for the submission of your research manuscript to our journal. I have now carefully assessed your manuscript, the referee reports and your revision plan, and I have discussed them with the editorial team. Based on our evaluation, we would like to invite you to revise your manuscript -as outlined in your revision plan- for potential publication in EMBO reports.

All referee concerns (as detailed in their reports) must be taken on board and addressed in a complete point-by-point response. Please also note that the conclusions of the study must be broadly supported by the provided results, and all possible (and alternative) models that could potentially explain the findings should be carefully considered and accurately described in the revised manuscript. 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 responses included in the next, final version of the manuscript.

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (December 2nd). Please discuss the revision progress ahead of this time with me if you require more time to complete the revisions.

*****IMPORTANT NOTE:
We perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:

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2) Your manuscript contains statistics and error bars based on n=2. Please use scatter blots in these cases. No statistics should be calculated if n=2.

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2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).
Please download our Figure Preparation Guidelines (figure preparation pdf) from our Author Guidelines pages https://www.embopress.org/page/journal/14693178/authorguide for more info on how to prepare your figures.

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines
()

6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here:

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in

a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

* NOTE - ITEMS BELOW TO BE INSERTED AT DISCRETION OF EDITOR

7) Please note that a Data Availability section at the end of Materials and Methods is now mandatory. In case you have no data that requires deposition in a public database, please state so instead of refereeing to the database.
See also < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

8) The journal requires a statement specifying whether or not authors have competing interests (defined as all potential or actual interests that could be perceived to influence the presentation or interpretation of an article). In case of competing interests, this must be specified in your disclosure statement. Further information: https://www.embopress.org/competing-interests

9) Figure legends and data quantification:
The following points must be specified in each figure legend:

- the name of the statistical test used to generate error bars and P values,
- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,
- the nature of the bars and error bars (s.d., s.e.m.)
- If the data are obtained from n {less than or equal to} 2, use scatter blots showing the individual data points.

Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.

See also the guidelines for figure legend preparation:
https://www.embopress.org/page/journal/14693178/authorguide#figureformat

- Please also include scale bars in all microscopy images.

10) We would also encourage you to include the source data for figure panels that show essential data.

Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available .

11) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at .

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I look forward to seeing a revised form of your manuscript when it is ready and please let me know if you have questions or comments regarding the revision.

You can use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Yours sincerely,

Ioannis Papaioannou, PhD

Editor
EMBO reports

Response to Reviewers

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

The manuscript by Neville et al addresses the link between the localization and the activity of the so-called "Pins complex" or "LGN complex", that has been shown to regulate mitotic spindle orientation in most animal cell types and tissues. In most cell types, the polarized localization of the complex in the mitotic cell (which can vary between apical and basolateral, depending on the context) localizes pulling forces to dictate the orientation.
The authors reexplore the notion that this polarized localization of the complex is sufficient to dictate spindle orientation, and propose that an additional step of "activation" of the complex is necessary to refine positioning of the spindle.

The experiments are performed in the follicular epithelium (FE), an epithelial sheet of cell that surrounds the drosophila developing oocyte and nurse cells in the ovarium. Like in many other epithelia, cell divisions in the FE are planar (the cell divides in the plane of the epithelium). The authors first confirm that planar divisions in this epithelium depends on the function of Pins and its partner mud, and that the interaction between the two partners is necessary, like in many other epithelial structures. Planar divisions are often associated with a lateral/basolateral "ring" of the Pins complex during mitosis. The authors show that in the FE, Pins is essentially apical in interphase and becomes enriched at the lateral cortex during mitosis, however a significant apical component remains, whereas mud is almost entirely absent from the apical cortex. Pins being "upstream" of mud in the complex, this is a first hint that the localization of Pins is not sufficient to dictate the localization of mud and of the pulling forces.
The authors then replace wt Pins, whose cortical anchoring strongly relies on its interaction with Gai subunits, with a constitutively membrane anchored version (via a N-terminal myristylation). They show that the localization of myr-Pins mimics that of wt-Pins, with a lateral enrichment in mitosis, and a significant apical component. Since a Myr-RFP alone shows a similar distribution, they conclude that the restricted localization of Pins in mitosis is a consequence of general membrane characteristics in mitosis, rather than the result of a dedicated mechanism of Pins subcellular restriction. Remarkably, Myr-Pins also rescues Pins loss-of-function spindle orientation defects.

They further show that the cortical localization of Pins does not require its interaction with Dlg (unlike what has been suggested in other epithelia). However, spindle orientation requires Dlg, and in particular it requires the direct Dlg/Pins interaction. The activity of Dlg in the FE appears to be independent from khc73 and Gukholder, two of its partners involved in its activity in microtubule capture and spindle orientation in other cell types.
Based on all these observations, the authors propose that Dlg serves as an activator that controls Pins activity in a subregion of its localization domain (in this case, the lateral cortex of

the mitotic FE cell).
They propose to test this idea by relocalizing Pins at the apical cortex, using Inscuteable ectopic expression. With the tools that they use to drive Inscuteable expression, they obtain two populations of cells. One population has a stronger apical that basolateral Insc distribution, and the spindle is reoriented along the apical-basal axis; the other population has higher basolateral than apical levels of Insc distribution, and the spindle remains planar. The authors write that Pins localization is unchanged between the two subsets of cells (although I do not entirely agree with them on that point, see below), and that although Mud is modestly recruited to the apical cortex in the first population, it remains essentially basolateral in both. In this situation, the localization of Insc in the cell is therefore a better predictor of spindle orientation than that of Pins or Mud. Remarkably, removing Dlg in an Insc overexpression context leads to a dramatic shift towards apical-basal reorientation of the spindle, suggesting that loss of Dlg-dependent activation of the lateral Pins complex reveals an Insc-dependent apical activation of the complex.
Overall, I find the demonstration convincing and the conclusion appropriate. One of the limitations of the study is the use of different drivers and reporters for the localization of Pins, which makes it hard to compare different situations, but not to the point that it would jeopardize the main conclusions. I do not have major remarks on the paper, only a few minor observations and suggestion of simple experiments that would complete the study.

Minor:

What happens to Pins and Mud in Dlg mutant cells that overexpress Insc and behave as InscA? Are they still essentially lateral, or are they more efficiently recruited to the apical cortex?

This is a terrific question. Of course we would love to know and intend to find out.

One way to do this (consistent with the manuscript) would be to generate flies that are *Dlg[1P20],* FRT19A/RFP-nls, hsflp, FRT19A; TJ-GAL4/+; Pins-Tom, GFP-Mud/UAS-Insc. (Note that these flies would only allow us to image Mud; we would have to repeat the experiment using GFP FRT19A; hsflp 38 to see Pins. This isn't ideal given that we'd like to image both together). Generating these flies is a major technical challenge because of the number of transgenes and chromosomes involved.

Our preferred way to do this would be to generate flies that are *Dlg[1P20]/Dlg[2]*; TJ-GAL4/+; Pins-Tom, GFP-Mud/UAS-Insc. So far, we've been unsuccessful. We find that the temperature-sensitive mutation *Dlg[2]* presents an activation barrier; while we are able to generate flies that are *Dlg[2]* / FM7 in combination with transgenes and/or mutations on other chromosomes, we do not always recover the *Dlg[2]* / Y males (which must develop at 18degrees) from these complex genotypes.

In the longer term (outside the scope of revision), we are working to develop more tools for imaging Mud and Pins that we hope will help answer this question.

Regarding the competition between Pins and Insc for dictating the apical versus basolateral localization of Insc, the Insc-expression threshold model could be easily tested in Pins62/62 mutants, where it is expected that only InscA localization should be observed, even at 25°C (unless Pins is required for the cortical recruitment of Insc, as it is the case in NBs - see Yu et al 2000 for example).

This is another great idea for an experiment and it's one we'd love to carry out. Again, the genetics are currently challenging, only because both UAS-Inscuteable and FRT82B *pins*^{p62} are on the third chromosome. We had another idea for testing the threshold model during revision, which was to repeat the experiment in which we express UAS-Insc in cells that are *Dlg*$^{IP20/IP20}$ at 25°C. In agreement with the model we found more Insc^{B} cells at the lower temperature, but there still weren't many; the rarity of the relevant cells (UAS-Insc OX in *Dlg* mitotic clones) limited our statistical power. We have added these data to Figure 6F and G. We added the following text to the manuscript: "We speculate that the two populations reflect different expression levels, meaning that there is a threshold of expression over which Inscuteable causes reorientation. Two lines of evidence support this possibility. Firstly, ectopic expression of Inscuteable in the embryonic ectoderm causes a reliable reorientation of spindle angles rather than a bimodal distribution, consistent with the possibility that the threshold is met in that tissue (Kraut et al., 1996; Bergstralh et al., 2015). Secondly, we found that spindle reorientation was less common when UAS-Inscuteable flies were maintained at 25°C rather than 29°C (Figure 6E,F). The UAS-GAL4 system is more efficient at the higher temperature."

I do not agree with the authors on P.10 and Figure 6A-D, when they claim that the apical enrichment of Pins is equivalent in both InscA and InscB cells. The number of measured cells is very low, and the ratio of apical/lateral Pins differs between the two sets of cells. The number of cells should be increased and the ratios compared with a relevant statistic method.

Absolutely fair. We have added more data (Figure 6D and Supplemental Figure 4D) and performed a statistical test (ANOVA). As the reviewer appears to have predicted, the overall trend in 6D softened so that the difference between apical and lateral Pins in InscB is not as strong as it initially appeared. However, there is not a statistical difference between either Mud or Pins localization in the Insc^{A} and Insc^{B} cells. (As expected, a statistical difference is observed for Insc localization.)

As the reviewer suggested, these data do not alter our main conclusions. However, we have amended the text to soften the initial claim: "*Instead, we find that Pins is apically enriched in both Insc^{A} and Insc^{B} cells, and this enrichment is similar in both populations (Figure 6A-D).*" has been changed to "*Instead, we find that Pins is apically enriched in both Insc^{A} and Insc^{B} cells (Figure 6A-D).*"

A lot of the claims on Pins localization rely on overexpression (generally in a Pins null background) of tagged Pins expressed from different promoters or drivers, and fused to different fluorescent tags. Therefore, it is difficult to evaluate to which extent the localization reflects an endogenous expression level, and to compare the different situations. As the cortical

localization of Pins relies on interaction with cortical partners (mostly GDP-bound Gai) which are themselves in limiting quantity in the cell, and in the case of Gai-GDP, regulated by Pins GDI activity, this poses a problem when comparing their distribution, because the expression level of Pins may contribute to its cortical/cytoplasmic ratio, but also to its lateral/apical distribution. Although I understand that the authors have been using tools that were already available for this study, I think it would be more convincing if all the Pins localization studies were performed with endogenously tagged Pins, even those with Myr localization sequences. In an age of CRISPR-Cas-dependent homologous recombination, I think the generation of such alleles should have been possible. Although this would probably not change the main claims of the paper, it would have made a more convincing case for the localization studies.

We don't disagree at all with this point. We did indeed try to stick with the published UAS-Pins-myr-GFP, not only for convenience but because it allows us to make comparisons to other studies using the same tool (Chanet *et al.* 2017 and Camuglia *et al.* 2022). Another consideration is that we used only one driver across our experiments (Traffic jam-GAL4). It is quite weak at the developmental stages that we examine, meaning that overexpression is not a major concern. (Indeed we have struggled with the opposite problem).

We certainly take the reviewer's comment seriously and we therefore described it in the manuscript. We are currently working to develop endogenous tools using CRISPR.

In response to the Reviewer's concern we've added the following paragraph to the discussion (under Limitations): *Another technical consideration is that our work makes use of transgenes under the control of Traffic jam-GAL4. While this strategy allows us to compare our results with previous work employing the same or similar tools, a drawback is that we cannot guarantee that Traffic jam-GAL4 drives equivalent expression to the endogenous Pins promoter (Chanet et al., 2017, Camuglia et al., 2022). However, given that Traffic jam-GAL4 is fairly weak at the developmental stages examined, we are not especially concerned about overexpression effects.*

The authors should indicate in the figure legends or in the methods that the spindle orientation measurements for controls for Pins62/62 are reused between figures 1, 3, 4, 5, 6 , and between figure 3, 4 and 5, respectively.

Absolutely. Added to the Methods section.

Reviewer #1 (Significance (Required)):

Altogether, this study makes a convincing case that the localization of the core members of the pulling force complex, Pins and Mud, is not entirely sufficient to localize active force generation, and that the complex must be activated locally, at least in the FE.
The notion of activation of the Pins/LGN complex has probably been on many people's mind for years: Pins/LGN works as a closed/open switch depending on the number of Gai subunits it interacts with, it must be phosphorylated, etc... suggesting that not all cortical Pins/LGN was active and involved in force generation. However the study presented here shows an interesting

case where localization and activation are clearly disconnected.
The authors show how Dlg plays this role in physiological conditions in the FE, and use ectopic expression of Insc to show that, at least in an artificial context, Insc can have the same "activating activity" (or at least an activating activity that is stronger than its apical recruitment capability and stronger than Dlg's activating activity). It is to my knowledge the first case of such a clear dissociation.
In their discussion, the authors are careful not to generalize the observation to other tissues. Although I did not reexplore all that has been published on the Pins/LGN-NuMA/Mud complex over the last 20 years, my understanding is that despite interesting cases of distribution of the complex like that of Mud in the tricellular junction in the notum, the localization model can still explain most of the phenotypes that have been described without invoking an activation step. If it is the case, then the activation model is another variation (an interesting one!) on the regulation of the core machinery, which are plentiful as the authors indicate in their introduction, and is maybe specific to the FE; if not, then it would be interesting to push the discussion further by reexamining previous results in other systems, and pinpointing those phenotypes that could be better explained with an activation step.

Overall, I find this is an elegant piece of work, which should be of interest to many cell and developmental biologists beyond the community of spindle orientation aficionados.

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

Summary:
The manuscript by Neville et al. addressed the mechanism how conserved spindle regulators (Pins/Mud/Gai/Dynein) control spindle orientation in the proliferating epithelia by revising "the canonical model", using the Drosophila follicular epithelium (FE). The authors examined the epistatic relationship among Pins, Mud and Dlg in FE and found that Pins controls the cortical localization of Mud by utilizing mutant analyses, and suggested their localization does not fully overlap using the newly generated knock-in allele. They also showed that Pins relocalization during mitosis depends on cortical remodeling, or passive model, where Pins localization changes with other membrane-anchored proteins. Their data further suggest that Pins cortical localization is not influenced by Dlg, but Pins-interacting domain of Dlg does affect spindle orientation. Based on these results, the authors propose that Dlg controls spindle orientation not by redistributing Pins, but by promoting (or "activating" from their definition) Pins-dependent spindle orientation. Interestingly, ectopic expression of Inscuteable (Insc) suggested that Insc localization, either apical or lateral, correlates with spindle orientation, and their localization is a dominant indicator of spindle orientation, compared to the localization of Pins and Mud, implicating potentially distinct roles of activation and localization of the spindle complex. Overall their genetic experiments are well-designed and provide stimulation for future research. However, their evidence is suggestive, but not conclusive for their proposal. I have several concerns about their conclusion and would like to request more detailed information as well as to propose additional experiments.

Major concerns:

1. This report lacks technical and experimental details. As the typical fly paper, the authors need to show the exact genotypes of flies they used for experiments. This needs to be addressed for Figures 1-6, and Supplemental Figures. Especially, which Gal4 drivers were used for UAS-Pins wt or mutant constructs in Figure 4 with pins mutant background, Khc73, GUKH mutant backgrounds. Which exact flies were used for mutant clone experiments for Supplemental Figure 3? (A for typical mosaic, and B for MARCM). Without these details, it is impossible to evaluate results and reproduce by others.

We take this concern very seriously!

- We listed the GAL4 driver (Traffic jam-GAL4) in the first section of the Materials and Methods: *Expression was driven by Traffic Jam-GAL4 (Olivieri et al., 2010).* The transgene and relevant citation have been added to Table 1.
- We explained the background stock for the MARCM experiment in the Materials and Methods: *Mosaic Analysis with a Repressible Cell Marker (after the method of Lee and Luo) was carried out using GFP-mCD8 (under control of an actin promoter) as the marker.* The transgene and relevant citation have been added to Table 1.
- In line with other fly studies (eg. Nakajima *et al.,* Nature 2013) and our own *Drosophila* work (Bergstralh *et al* Current Biology 2013, Bergstralh*, Lovegrove*, St Johnston NCB 2015, Bergstralh *et al* Development 2016, Finegan *et al* EMBO J 2019, Cammarota*, Finegan* *et al* Current Biology 2020) we were careful to show the relevant genotype components in each figure.
- We included a fully referenced Supplementary Table (Table 1 – *Drosophila* genetics) listing every mutant allele or transgene with a citation and a note about availability. We have expanded this table in response to the author's concern (see above).

2. Related to the comment 1, how did the author perform "clonal expression of Ubi-Pin-YFP" in page 5? As far as I understand, Ubi-Pin-YFP is expressed ubiquitously by the ubiquitin promoter.

The reviewer makes a good point. We regret that we did not make this experiment more clear. Ubi-Pins-YFP was recombined onto an FRT chromosome (FRT82B). We made mitotic clones.

We have clarified this in the Methods section as follows: "Mitotic clones of Ubi-Pins-YFP were made by recombining the Ubi-Pins-YFP transgene onto the FRT82B chromosome"

3. In page 6, if Pins relocalization is passive and is associated with membrane-anchored protein remodeling during mitosis, its relocalization can be suppressed by disrupting the process of mitotic remodeling (mitotic rounding). The authors should test this by either genetic disruption or pharmacological treatment for the actomyosin should cause defects in Pins relocalization, which bolster their conclusion.

We agree that this is a cool idea for an experiment, but we don't know (or claim) that mitotic rounding and membrane-anchored protein remodeling during mitosis are inextricably linked.

Notably, the remodeling we describe reflects cell polarity; apical components are evidently moved to the lateral cortex. This is contrary to understanding of rounding, which reflects isotropic actomyosin activity (Chanet et al., 2017. & Rosa et al., 2015). Therefore we don't understand what a "negative" result would mean, or for that matter that a "positive" result would be safe to interpret.

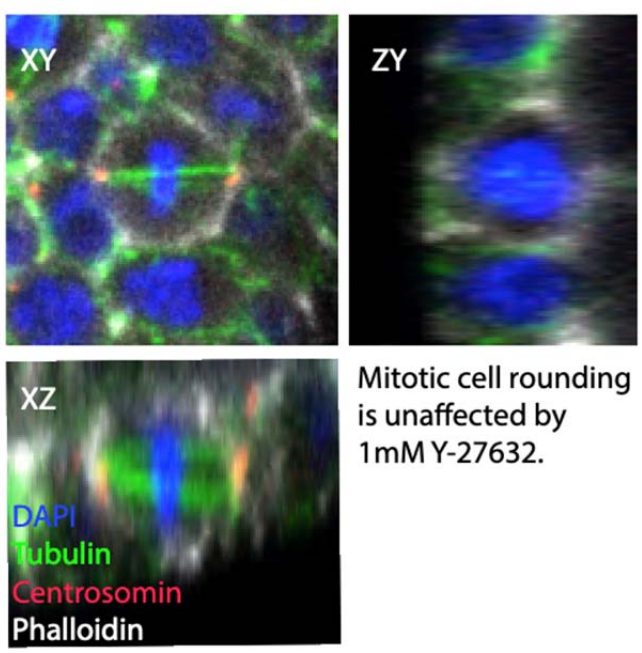

Figure 1- The follicular epithelium shows not phenotypic effect on cell rounding when injected with 1mM Y-27632.

We have attempted many strategies to prevent cell rounding in the follicular epithelium, none of which have successfully prevented rounding. 1) We attempted to genetically knockdown Moesin in the FE and did not see an effect on cell rounding. However we couldn't confirm knockdown and therefore are not confident in this manipulation. 2) It is difficult to interpret the result of genetically disrupting Myosin, because it causes pleiotropic effects, such as inhibition of the cell cycle, and disruption of monolayer architecture. 3) We treated egg chambers with Y-27632 (a Rok-inhibitor) and examined its effect on mitotic cell rounding and on cytokinesis, which are Rok-dependent processes. Our experiments were performed using manually-dissociated ovarioles treated for 45 minutes in Schneider Cell Medium supplemented with insulin. Even at our maximum concentration of 1mM Y-27632, several orders of magnitude above the K_i, we are unable to see any effect on mitotic cell shape or actin accumulation at the mitotic cortex and did not observe any evidence of defective cytokinesis (Figure 1). We also did not observe defects in spindle organization or orientation, as would be expected from failed rounding. We therefore do not believe that the inhibitor works in this tissue. One possible explanation is that the follicle cells are secretory, and likely to pass molecules taken up from the media quickly into the germline. Therefore, we do not anticipate that we can perform this experiment to our satisfaction.

4. The critical message in this manuscript is that the core spindle complex mediated by Pins-Mud controls spindle orientation by "activation", but not localization. The findings that Pins and Mud localization is not influenced by Insc and that ecotpic Insc expression and genetic Mud depletion (Figure 6) might support their proposal, but these results just suggest their localization does not matter. I wonder how the authors could conclude and define "activation". What does this activation mean in the context of spindle orientation? Can the authors test activation by enzymatic activity or assess dynamics of spindle alignment?

We intend for the critical message of the manuscript to be that "The spindle orienting machinery requires activation, not just localization." We absolutely do not make the claim that localization is

not important, only that it is not sufficient. The reviewer recognizes this point here and in a subsequent comment: "The authors showed that Pins and Mud localization themselves are not sufficient for the control of spindle orientation with genetic analyses."

We also do not claim that Pins and/or Mud localization are not impacted by Inscuteable. On the contrary, we plainly see and report that they are; the intensity profiles in Figure 6 are distinct from those in Figure 2, as discussed in the text.

We appreciate the reviewer's point about activation. Since we do not understand these proteins to be enzymes, we aren't sure what enzymatic activity would be tested. The dynamics of spindle alignment in this slowly developing system are prohibitively difficult to measure: the mitotic index is very low (~2%) and only a very small fraction of those cells will be in a focal plane that permits accurate live imaging in the apical-basal axis. Alternative modes of activation include conformational change and/or a connection with other important molecules. The simplest possibility would be that Dlg allows Pins to bind Mud, but so far our data do not support it. We have added the following paragraph to our discussion:

The mechanism of activation remains unclear. While the most straightforward possibility is that Dlg promotes interaction between Pins and Mud, our results show that Mud is recruited to the cortex even when Dlg is disrupted (Figure 4D). Alternatively, Discs large may promote a conformational change in the spindle-orientation complex and/or a change in complex composition. Furthermore, the Inscuteable mechanism is not likely to work in the same way. Dlg binds to a conserved phosphosite in the central linker domain of Pins and should therefore allow for Pins to simultaneously interact with Mud (Johnston et al., 2009). Contrastingly, binding between Pins and Inscuteable is mediated by the TPR domains of Pins, meaning that Mud is excluded (Culurgioni et al., 2011; 2018). While a stable Pins-Inscuteable complex has been suggested to promote localization of a separate Pins-Mud-dynein complex, our work raises the possibility that it might also or instead promote activation.

5. In page 7-8, although Pins-S436D rescue spindle orientation, but Pins-S436A does not in pins null clones background, Pins localization is not influenced by Dlg. This questions how exactly Pins and Dlg can interact, and how Dlg affect Pins function. Related to this observation, in the embryonic Pins:Tom localization in dlg mutant does not provide strong evidence to support their conclusion given the experimental context is different from previous study (Chanet et al., 2017).

We agree with the reviewer. Our data (this paper and previous papers) and the work of others indicate that this interaction is important for spindle orientation (Bergstralh et al., 2013a; Saadaoui et al., 2014; Chanet et al., 2017). However, we show here that Dlg doesn't obviously impact Pins localization (as proposed in our earlier paper), but does impact the ability of the spindle orientation machinery to work (hence activity).

The reviewer makes a very good point. Our experimental context is different from the previous study concerning Pins and Dlg in embryos: Chanet *et al* (2017) performed their work in the

embryonic head, whereas we look at divisions in the ventral embryonic ectoderm (Figure 2A). These are distinct mitotic zones (Foe V., 1989) and exhibit distinct epithelial morphologies. We have elaborated on our findings with additional data from embryos. We examined spindle angles in Dlg^{1P20}/Dlg^{2} mutant embryonic tissue by looking at embryos from Dlg^{1P20}/Dlg^{2} mothers (Figure 4I). We found that there is a significant spindle orientation defect in these embryos. Therefore, Dlg impacts spindle orientation independently of the localization of Pins to cell cortices in the follicular, and early embryonic ectoderm.

We also examined the relationship between Pins and Dlg in a different epithelium, the ventral ectoderm of the early (Stage 10) *Drosophila* embryo. Consistent with our findings in the FE, mitotic spindles in this tissue are misoriented with respect to the apical-basal plane in embryos from Dlg^{1P20}/Dlg^{2} mothers (Fig 4H). As in the FE, Pins:Tom is observed at mitotic cell cortices, indicating that Pins localization in this tissue does not require direct interaction with Dlg (Fig 4I). These results contrast with the finding that cortical localization of Pins in embryonic head epithelial cells is lost when *Dlg* mRNA is knocked down (Chanet et al., 2017). We therefore speculate that Dlg plays an additional role in that tissue, upstream of Pins.**"**

6. In page 11, the authors state "... that activation of pulling in the FE requires Dlg". I was not convinced by anything related to "pulling". There is no evidence to support "pulling" or such dynamic in this paper, just showing Mud localization, correct?

We appreciate the reviewer's concern. The original sentence read that "*We interpret [our data] to mean that interaction between Pins and Dlg, which is required for pulling, stabilizes the lateral pulling machinery even if Dlg is not a direct anchor*." This statement is based on work across multiple systems, including the *C. elegans* embryo (Grill *et al* Nature 2001), the *Drosophila* pupal notum (Bosveld *et al,* Nature 2016), and HeLa cells (Okumura *et al eLife* 2018), which shows that Mud/dynein-mediated pulling (on astral microtubules) orients/positions spindles. This is described in the introduction.

To address the reviewer's particular concern, we have replaced "pulling" with "spindle-orientation machinery," so that this sentence now reads …"activation of the spindle-orientation machinery in the FE requires Dlg."

7. Ectopic expression of Insc (Figure 6) provided a new idea and hypothesis, but the conclusion is more complicated given that Insc is not expressed in normal FE. For example, the statement that "Inscuteable and Dlg mediate distinct and competitive mechanism for activation of the spindle-orienting machinery in follicle cells" is probably right, but it does not show anything meaningful since Insc does not exist in normal FE. Is Dlg in a competitive situation during mitosis of FE? If so, which molecules are competitive against Dlg? The important issue is to provide a new interpretation of how spindle orientation is controlled epithelial cells. I strongly recommend to add models in this manuscript for clarity.

We considered the addition of model cartoons very carefully in preparing the original manuscript, and again after review. Our concern is that model figures would obscure rather than clarify the message. As the reviewer points out, we do not understand how activation works,

and as discussed in the manuscript we don't think it's likely to work the same way in follicle cells (Dlg) as it does in neuroblasts (Insc). Therefore model figure(s) are premature.

We do not agree with the statement that "Inscuteable and Dlg mediate distinct and competitive mechanism for activation of the spindle-orienting machinery in follicle cells… does not show anything meaningful." This is a remarkable finding because it suggests that there is more than one way to activate Pins. Given the critical importance of spindle orientation in the developing nervous system, and the evolutionary history of the Dlg-Pins interaction, we think that this finding supports a model in which the Dlg-Pins interaction evolved in basal organisms, and a second Inscuteable-Pins interaction evolved subsequently to support neural complexity. These ideas are raised in the Discussion.

The reviewer also writes that "The important issue is to provide a new interpretation of how spindle orientation is controlled epithelial cells." We find this concern perplexing, since the reviewer clearly recognizes that we have provided a new interpretation: Dlg is not a localization factor but rather a licensing factor for Pins-dependent spindle orientation.

Minor comments:
8. Some sections were not written well in the manuscript. "It does not" in page 6. "These predictions are not met". I just couldn't understand what they stand for. Their writing has to be improved.

We prefer to retain the original language, which in our opinion is fairly clear. Our study is hypothesis-driven and based on assumptions made by the current model. We used direct language to help the reviewer understand what happened when we tested those assumptions.

9. In page 9, Supplementary Figure 4 should be cited in the paragraph (A potential strategy for..), not Supplemental Figure 1A, and 1B.

Good catch, thank you! We have corrected this.

10. In page 10, the authors examine aPKC localization in Insc expressing context of FE. Does aPKC localization correlate with Insc localization (Insc dictates aPKC?)? aPKC is not involved in spindle orientation from the author's report (Bergstralh et al., 2013), so it does not likely provide any supportive evidence.

I'm afraid we don't entirely understand this comment. The interdependent relationship between aPKC and Inscuteable localization is long-established in the literature and was previously addressed in the follicle epithelium (Bergstralh et al. 2016). We do not make the claim here that aPKC governs spindle orientation. We are emphasizing that the difference between InscA and InscB cells extends to the relocalization of polarity components involved in Insc localization. As described in the manuscript, these data are provided to support our threshold model:

“In agreement with interdependence between Inscuteable and the Par complex, we find that aPKC is stabilized at the apical cortex in Insc^{A} cells but enriched at the lateral cortex in Insc^{B} cells (Figure 6E). This finding is consistent with an Inscuteable-expression threshold model; below the threshold, Pins dictates lateral localization of Inscuteable and aPKC. Above the threshold, Inscuteable dictates apical localization of Pins and aPKC.”

11. In Dicussion page 12, "In addition, we find that while the LGN S408D (Drosophila S436D) variant is reported to act as a phosphomimetic, expression of this variant has no obvious effect on division orientation (Johnston et al., 2012)". Where is the evidence for this? I interpret that this phosphomimetic form can rescue like wt-Pins not like unphospholatable mutant S436A, so it means that have an effect on spindle orientation, correct?

The reviewer makes a good point. We regret the confusion. We mean to point out that the S436D variant is no different from the wild type. We have amended the text to clarify:

“In addition, we find that while the LGN S408D (Drosophila 436D) variant is reported to act as a phosphomimetic, this variant does not cause an obvious mutant phenotype in the follicular epithelium (Johnston et al., 2012). What then is the purpose of this modification? Since the phosphosite is highly conserved through metazoans, one possibility to consider is that the phosphorylation regulates the spindle orientation role of Pins, whereas unphosphorylated Pins plays a different role (Schiller and Bergstralh, 2021).”

Reviewer #2 (Significance (Required)):

The authors showed that Pins and Mud localization themselves are not sufficient for the control of spindle orientation with genetic analyses. While the authors tried to challenge the concept of "canonical model", there is no clear demonstration of "activation" of the spindle complex. I appreciate their genetic evidence and new results, and understand the message that Pins and Mud effects are beyond localization, but there is no alternative mechanism to support their model. At the current stage, their evidence provides more hypothesis, not conclusion. Based on my expertise in Developmental and Cell biology, I suggest that the work has an interest in audience who studies spindle machinery, but for general audience.

We think that the reviewer fundamentally shares our perspective on the study. Our work tests assumptions made by the canonical model and shows that they aren’t always met (meaning that the question of how spindle orientation works in epithelia at least is still unsolved), and that in the FE at least one component (Dlg) has been misunderstood. We reach a major conclusion, which is that localization of Pins is not enough to predict spindle orientation in the FE.

It’s absolutely true that the precise molecular role of Dlg has not been solved by our study. This is a major question for the lab, and we are currently undertaking biochemical work to address it. It’s probably more work than we can (or should) do on our own, which is just one reason to share our current results with colleagues.

One fundamental reason for undertaking this study is that 25 years of spindle orientation studies released into an environment in which “positive” conclusions are the bar for publication success may have burdened the field with claims that are overly-speculative. We appear to have contributed to this problem ourselves in 2013. With that in mind we contend that providing an alternative molecular mechanism at this point is premature and would impair rather than improve the literature.

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

Neville et al re-examine the role and regulation of Pins/LGN in Drosophila follicular epithelial cells. They argue that polar or bipolar enrichment of Pins localisation at the plasma membrane is not crucial for spindle orientation, and therefore propose that Pins must be somehow activated to function. These interpretations are not supported by the data. However, the data strongly suggest an alternative interpretation which is of major biological significance.

As an initial point, we disagree with the summary above. We do not argue that enrichment of Pins is not crucial for spindle orientation. We argue that enrichment of Pins is not sufficient. This is why we titled the paper “The spindle orienting machinery requires activation, not just localization” instead of “The spindle orienting machinery requires activation, not localization.”

Although we disagree with reviewer, we appreciate their criticism of our manuscript and are glad for the opportunity to clarify our findings. In our responses to the specific comments (below) we explain why our data contradict the reviewer’s model and how we used their comments to strengthen the manuscript.

Comments:

1. In the experiments on Dlg mutants (Fig 4D, S3) visualising Pins:Tom, the wild-type needs to be shown next to the Dlg mutant image, otherwise a comparison cannot be made. For example, Pins:Tom looks strongly enriched at the lateral membranes in the wild-type shown in Fig 2B&C, but much more weakly localised at the lateral membranes in Dlg1P20/2 mutants in Fig 4D. Thus, it looks like the Dlg GUK domain is required for full enrichment of Pins:Tom at lateral membranes, even if some low level of Pins can still bind to the plasma membrane in the absence of the Dlg GUK domain. Quantification would likely show a reduction in Pins:Tom lateral enrichment in the Dlg1P20/2 mutants - consistent with the spindle misorientation phenotype in these mutants.

The reviewer raises a reasonable concern about Figure 4D. We noted the difficulty of imaging Pins:Tom, which is exceedingly faint, in our original manuscript. For technical reasons, only one copy of the transgene was imaged in the experiment presented in 4G (two copies were used in Figure 2B), and the lack of signal presented an even greater challenge. In the manuscript we went with the clearest image. To address the reviewer’s concern, we have added signal intensity plots to this figure showing that Pins:Tom and Pins-myr are both laterally enriched at

mitosis in *Dlg[1P20]/Dlg[2]* mutants. These data have been added as a new panel (E) in Figure 4. We were also able to replace the pictures in 4D with new ones generated after review.

2. In Fig 4E, the phosphomutant PinsS436A-GFP looks more strongly apical and less strongly lateral than the wildtype Pins-GFP, consistent with the spindle misorientation phenotype in S436A rescued pins mutants.

The reviewer has an eagle eye! We did not detect a difference in localization across the three transgenes, though we were certainly looking for it (that's why we generated these flies in the first place). Again, the strength of signal was a major challenge in these experiments, and we therefore went with the cleanest image. In response to the reviewer's concern, we note that the S436A and S436D examples shown have equivalent apical signal, but only the S436A fails to rescue spindle orientation.

Together, Reviewer Comments 1 and 2 suggest a model in which Dlg is required for lateral enrichment of Pins at mitosis. As described in the manuscript, this is the very model proposed in our own previous study (Bergstralh, Lovegrove, and St Johnston; 2013), and reiterated in a subsequent review article (Bergstralh, Dawney, and St Johnston; 2017). We point these publications out because the senior author of the current manuscript is not especially enthusiastic about showing himself to be wrong (twice!) in the literature. He therefore insisted on seeing multiple lines of evidence before making the counterargument:

1) The reviewer's model (the 2013 model) is firstly challenged by work shown in Figure 3. We find that membrane-anchored proteins (even just myristoylated RFP!) demonstrate lateral enrichment at mitosis, regardless of whether or not they interact with the Dlg-GUK domain.
2) Even stronger evidence is shown in Figure 4F. Pins-myr-GFP is very plainly enriched at the lateral cortex in *Dlg[IP20]/Dlg[2]* mutant cells (now demonstrated with signal intensity plots in FIGURE 4E). However, the spindle doesn't orient correctly (quantified in 4C). Since Dlg is impacting spindle orientation independently of Pins localization, these data support our "claim in the final sentence of the abstract '*Local enrichment of Pins is not sufficient to determine spindle orientation; an activation step is also necessary*'."

3. In the InscA examples, Pins:Tom looks apical. In the InscB examples, Pins:Tom looks more laterally localised, consistent with the spindle orientations in these experiments.

These figures (6A-D) don't only show/quantify Pins:Tom localization. They also show localization of GFP-Mud. Whereas Pins:Tom is certainly apically enriched in the InscA examples, the interesting finding is that GFP-Mud is not. In strong contrast, it instead shows a weak apical localization and a strong lateral enrichment. As described in the manuscript, this pattern of Mud localization predicts normal spindle orientation, which is not observed in these cells.

Thus, these data appear to support the existing model that Pins enrichment at the plasma membrane is a key factor directing mitotic spindle orientation in these cells. The author's claim in the final sentence of the abstract "Local enrichment of Pins is not sufficient to determine spindle orientation; an activation step is also necessary" is not supported by the data.

We are pleased that the reviewer shared this quote; our claim is that Pins localization is not sufficient, not that it is unnecessary (see above). We absolutely do not dispute that "Pins enrichment at the plasma membrane is a key factor directing mitotic spindle orientation."

The open question posed by the data is why GFP-Mud is excluded apically & basally during mitosis, while Pins:Tom is not. The simple alternative model is that Mud only localises to the plasma membrane where Pins is most strongly concentrated, such that Mud strongly amplifies any Pins asymmetry. Thus, even myr-Pins can still rescue a pins n mutant, because myr-Pins is still enriched laterally compared to apically (or basally).

Thus, I would strongly suggest re-titling the manuscript to: "Mud/NuMA amplifies minor asymmetries in Pins localisation to orient the mitotic spindle".

Well, that is a good-looking title, and we're therefore sorry to decline the suggestion. However, as described above, Figure 4D shows that Pins enrichment does not always predict spindle orientation. More importantly, Figure 6A (cited by the reviewer in Comment 3) very plainly shows that Mud does *not* "only locali[ze] to the plasma membrane where Pins is most strongly concentrated." In this picture – and across multiple InscA cells (Figure 6B) - Pins is strongly concentrated at the apical surface, whereas Mud is not.

Mud/NuMA presumably achieves this amplification by binding to the plasma membrane only where Pins is concentrated above a critical threshold level. This would mean a non-linear model based on cooperativity among Pins monomers that increases the binding avidity to Mud above the threshold concentration of Pins monomers.

This is essentially a minor revision of the standard model, which we expected would hold true in the FE. As described above, it is not supported by our data.

Reviewer #3 (Significance (Required)):

The manuscript is focused on the question of mitotic spindle orientation in epithelial cells, which is a fundamental unsolved problem in biology. The data reported are impressive and important, providing new insights into how the key spindle orientation factors Mud/NuMA and Pins/LGN localise during mitosis in epithelia. I recommend publication after major revisions.

We are delighted that the reviewer finds our data impressive and important, and our experiments insightful. We understand that the "major revisions" requested are meant to bring the paper in line with their model (our own 2013 model). Since the data in the manuscript contradict that model, we focused our revisions on clarifying and strengthening the message.

Dear Dan,

Thank you for the submission of your revised manuscript to EMBO reports and for your patience during its peer review. We have now received the reports from two of the original referees who agreed to re-evaluate your study.

Both referees find that the study was significantly improved during revision and that most concerns of all three referees were successfully addressed, and they now support publication of the manuscript. They request only few minor improvements (please see their comments appended below), which I need you to address before I can accept the manuscript. Please make sure that the changes are highlighted (or "tracked") to be clearly visible in the revised manuscript file.

From the editorial side, there are also a few things that we need before we can proceed with the official acceptance of your manuscript:

- The title should be short (up to 100 characters including spaces), informative, and accurate. I would recommend revising it to "The mitotic spindle orienting machinery of Drosophila requires activation, not just localization". Please revise it in your manuscript file accordingly if you agree with the suggested changes.

- The heading "Summary" needs correcting to "Abstract".

- The abstract should be a single paragraph describing all key novel findings of the study, written in present tense, and it should not exceed 175 words. Please revise it accordingly.

- Please provide up to 5 keywords in your revised manuscript.

- According to our journal's policy, "data not shown" (stated on page 10 of your manuscript) is not permitted. All data referred to in the paper should be displayed in the main or Expanded View figures, or in the Appendix. Please add these data or change the text accordingly if these data are not central to the study and its conclusions.

- Your Figure legends have been inspected by our data editors for completeness and accuracy. Please see the required changes in the attached Word file and address all comments in your revised manuscript (with tracked changes).

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- There is also a callout to "Supplementary Figure 6D" which does not exist, please correct.

- Please update your competing interests statement: the heading should be "Disclosure and competing interests statement" and the statement "The authors declare that they have no conflict of interest.", since you have no competing interests to declare.

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We would also welcome the submission of cover suggestions or motifs to be used by our Graphics Illustrator in designing a cover.

We look forward to seeing a final version of your manuscript as soon as possible.

Best regards,

Ioannis

Ioannis Papaioannou, PhD
Editor
EMBO reports

Referee #1:

I only had minor comments on the original version of the manuscript. The authors have addressed some of these comments, and provide reasonable technical explanations for not addressing the others in the timeframe of the revision.
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We're happy to take your suggestion. I used "The *Drosophila* mitotic spindle orientation machinery…" because I think it looks a little neater, but I'm fine with your version.

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Done.

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The data have been published in a previous article. We simply removed the statement.

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Please send us this information along with your revised manuscript.

A) The canonical model for spindle orientation in the *Drosophila* follicular epithelium and embryonic ectoderm is incomplete. Spindle orientation in the apical-basal axis relies not only on the spatial localization of Pins but also interaction with a partner protein.
B)

- Pins location, which is sometimes used as a proxy for the spindle orienting machinery, may not always be sufficient to predict either Mud localization or spindle orientation.
- Spindle orientation in some epithelia relies on interaction between Pins and Discs large, but this interaction does not explain lateral localization of Pins during mitosis.
- The reorienting effect of ectopically-expressed Inscuteable is Dlg-independent.

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We agree and have made this edit.

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Comments on the revised manuscript by Neville et al. for EMBO Reports

I reviewed the revised manuscript and found that the authors addressed the major issues. I thought the quality of the work improved, but the authors did not fully address some reviewer's concerns or include important information regarding the resources. I thus request the further round of revision that address the following issues (including the comment by the reviewer #3).

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We have added a new Table (EV2) that includes genotypes for each experiment.

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authors most likely generated for this study. I am not aware of the way the authors used to generate this fly stock, but the sequence of the plasmid and the exact method needs to be shown in detail.

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We are a bit stymied. We took the concern of Reviewer #3 seriously in preparing our revision. The wild type images are in the paper already, but used in earlier figures to make a necessary argument. We are therefore being asked to repeat the same images in later figures. We feel this would be a confusing choice and take up unnecessary space. The signal quantification added during revision A) obviates this problem and B) is more meaningful than single images anyway.

***
Rev_Com_number: N/a
New_manu_number: EMBOR-2022-56074V2
Corr_author: Bergstralh

Dr. Dan Bergstralh
University of Rochester
Department of Biology
Hutchison Hall
University of Rochester
Rochester, New York 14627
United States

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Ioannis

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This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your manuscript.
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Abridged guidelines for 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.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots 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. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data Presentation.

2. Captions

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

- a specification of the experimental system investigated (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.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

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Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Yes	Materials and Methods, Table 1 Drosophila Genetics

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DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Not Applicable	

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Not Applicable	
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Not Applicable	
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

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Animal observed in or captured from the field: Provide species, sex, and age where possible.	Not Applicable	
Please detail **housing and husbandry conditions**.	Yes	Materials and Methods

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Not Applicable	

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Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Figure Legends
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Not Applicable	
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Figure Legends and Materials and Methods

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Figure Legends
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Figure Legends

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Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: 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.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Not Applicable	
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
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Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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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.	Not Applicable	

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Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	