Microtubule-dependent pushing forces contribute to long-distance aster movement and centration in Xenopus laevis egg extracts

Taylor Sulerud, Abdullah Sami, Guihe Li, April Kloxin, John Oakey, and Jesse Gatlin

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)
RE: Manuscript #E20-01-0088
TITLE: Microtubule-dependent pushing forces are sufficient for long-distance aster movement and centration in Xenopus laevis egg extracts

Dear Jay

As you will see both reviewers find your work interesting and important but raise some significant concerns that need to be addressed prior to publication. In general there is fairly good consensus between the two reviewers regarding the necessary additional experiments to further clarify the role of dynein as well as the technical limitations of the hydrogel system that need to be acknowledged. In addition, both reviewers point out the need for reconciliation of some key work by others, which is important to provide clarity to your work and that of others. I am hopeful that you will be able to address the concerns with additional experimentation and submit a revised manuscript that addresses these points.

Claire

Monitoring Editor
Molecular Biology of the Cell

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Dear Dr. Gatlin,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

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Please contact us with any questions at mboc@ascb.org.

Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

E20-01-0088 Sulerud et al., and Gatlin
As the authors explain in their great introduction, the position of the astral MT network in cells, specifically its center, determines the eventual location of the spindle apparatus and ultimately the cytokinetic furrow. Positioning of the MT aster often results in its movement to the center of a cell how these forces responsible for this movement are generated and then integrated within cells of varied sizes and geometries remains an open question.

This paper overall addresses this interesting question using innovative methods. The photocontrolled hydrogel system will be of interest to a broad audience of physical biologists. The major conclusion of the paper is adequately supported by the data, with the caveats below.. The paper is well suited for MBoC if important revisions are made.

Major concerns
All the experiments are performed in egg extract where F-actin is depolymerized with a drug this is quite different from eggs. This will have a major effect on the mechanics of aster movement relative to intact eggs. This is ok, this is a standard system. But this central point must be clearly flagged for readers ideally in the abstract or intro and as a clear statement in conclusion. As it stands now, only researchers very familiar with Xenopus extract would be aware of this.

The experiment in Figure 4 is mis-interpreted or over-interpreted. Microtubules turn over rapidly throughout interphase asters. Therefore, the experiment reported does NOT uncouple MT growth from MTOC movement. If the authors present high resolution speckle tracking as fiducials on individual they might be able to propose this interpretation. But even then, this interpretation is questionable. Fig 4 is not new information relative to previous figures and should move to a supplement.
The authors state that their hydrogel is "compatible" with Xenopus extracts referencing Bisht et al., 2019. This is perhaps technically correct. However, that paper flowed extract into devices with hydrogel posts and looked at nuclear formation and import. It did not look at microtubule structures and cell cycle dynamics. More problematic is that embedding extract in a hydrogel which is done in the experiments here is very different from flowing it around posts and the majority of data in the paper is from extracts in hydrogels. It is clear in multiple figures that MT density is lowered in hydrogel experiments, perhaps unpredictably. The authors should admit about technical limitations in paragraph one of their discussion.

p150 CC1 is a protein inhibitor of the dynein motor. Addition of p 150 CC1 to extract (Figure1B) does not affect aster centration -this is a strong experiment and would seem to favor a pushing model but it was only done in extracts while all the rest of experiments in the manuscript are performed in hydrogel. The conclusions of the paper would be better supported if one hydrogel experiment was performed with p150 CC1.

Overall, this reviewer would like to see need to see more objective evaluation of strengths and limitations of the new methods.

Minor concerns

Ref is missing from the top of page 5 aster positioning in large blastomeres (ref), aster movement away...

Good 2018 ref mentioned in the paper is not in the reference list.

I think the point of Figure 2 is asters do not center when barriers are asymmetrical, but I think the geometries are rather hard to interpret and I am not certain how they relate to pushing.

Comparing a single geometry plus/minus CC1 could be more informative. I think the geometry in Figure 2A makes more sense than in Figure 2B. The lobster trap is interesting but hard to interpret. With respect to pushing or pulling one could say the aster cannot go through the restricted space because the aster as a whole is too large.

In Figure 3 I agree that faster movement away a more focused V-shaped barrier might be consistent with pushing. The less focused V-shape end up with more MT in front-if there was cytoplasmic pulling one would think the aster might move faster. However, this reviewer is not sure of the exact shape of the chamber. Are all the microtubules in front of the bead in the wide V-shape hitting a wall and slowing the aster down. I think this experiment could really benefit from p150 CC1 addition.

In Figure 4, why did authors change to yet another configuration? Aster moves faster in teardrop situation-does that always happened? How do the authors interpret this?

Reviewer #2 (Remarks to the Author):

Understanding if MTs predominantly push or pull using dynein forces to center centrosomes nuclei or spindles is an important aspect of cell division and polarity. Many initial in vitro work from Leibler/Dogterom groups used purified MTOC and MTs in small microchambers, supporting that pushing can move an aster, but the direct relevance to in vivo situations has been limited. In part
because in cells, apart from few exceptions like the small fission yeast, work from many groups, in models like C. elegans, zebrafish, Xenopus or mammalian cells have long supported that centrosome centration requires dynein activity, suggesting that MT pulling from the cortex or cytoplasm is likely dominant. Here, the authors approach this problem, using a tractable Xenopus extract embedded in large ~100μm size chambers, made of PDMS or a neat photo-polymerizable hydrogel technology that even allows to change aster confinement in real-time. Their result suggest that in contrary to the established dogma in vivo, MT pushing may be the main force that move and center asters, even if MTs are long, presumably because in large asters MTs may bundle or branch which could limit buckling. If correct, this finding could be important in the field.

Although I am impressed by the technology, and some of the videos, there are two major limits in the current MS, that need to be fully addressed before I could recommend publication: (i) the evidence for pushing vs pulling may be prone to caveats in the assays and interpretation; and (ii) the text is worded in a rather defensive manner omitting or mis-citing some very important literature that goes against the author's conclusion, giving the reader the sensation of a forced message.

(i) The main evidence supporting pushing is based on the addition of p150-CC1 glue, a potent dynein inhibitor, which does not alter aster centration in a PDMS chamber (Fig 1B). There are plausible caveats and no appropriate controls for this essential result (apart from a mitotic extract in a very different imaging set-up in supp). 1) The use of PDMS is worrying. PDMS can rapidly deplete many proteins or drugs, and thus the authors need to directly provide a control for the lack of dynein activity while imaging centering asters in their PDMS chambers. This could be done by imaging ER/mitochondria retrograde flow as done in a recent paper by the Ferrell lab in similar extracts (Cheng, et al. Science 2019). 2) The authors use TMBD as a marker for MTs throughout the paper, and there is no appropriate control for it. TMBD is derived from tau which is a strong MT stabilizer. In the hands of many labs, TMBD can create an over-stabilization of MT structures, and exhibit photo-toxic effects which bundle asters or create bizarre spiral arrangements, much like those seen in Figure 1C. Thus, there is a probability that TMBD could mask the effect of adding p150-CC1 glued, or more importantly that it directly promotes MT pushing by stabilizing them. Leaders in the field (see many recent papers from Ferrell or Mitchison labs on Xenopus based MT asters in vitro) use labelled tubulin or sir-tubulin, as better and more established probes. This defines the state of the art on which the authors should base a large set of required controls. I am willing to believe the authors’ claim but I would be relatively strict with seeing a set of very clean controls, because dynein inhibition experiments are IMHO the sole support for the general claim and title of this paper.

Indeed, the interpretation of the subsequent findings based on pushing is rather shaky. The sawtooth pattern in figure 2A may not be a very relevant support for pushing because MTs appear aligned along the long axis of the barrier, rather than pushing “end-on” onto the little negative curves. The difference in speeds in the open triangles in fig 3B and 3C could just emerge from a larger drag of the aster in 3C, as evident from the fact that they slow down as they grow, with little to do with angles of contact between MTs and chamber walls. Finally, the stop of the aster at the lobster trap entrance, could simply result from an effective stiffness of the dense MT network around the centrosome that prevents it from moving into the trap.

(ii) Another important issue, is that this work contradicts recent essential literature by eminent experts in the field which are either surprisingly not cited, or mentioned so as to give the sensation that all experiments that contradict the author’s findings are incorrect! This is not proper, and the MS needs significant rewording, to address discrepancies in a more honest/transparent manner, avoiding this systematic bias. One crucial omitted paper is from Wurh and Mitchison (Curr. Biol 2010), which clearly demonstrate the requirement of dynein using the same p150-CC1 inhibitor in
Xenopus eggs in vivo. More dramatically, Cheng and Ferrel published a recent paper (Cheng Science 2019), which is also not cited, using very similar cytoplasmic interphase Xenopus extracts, which provided remarkable and very clean demonstration of the requirement of dynein activity for nuclei and centrosomes centration in large asters. How should one reconcile these findings with the one presented here? These are the Xenopus papers that the authors should confront their data with, rather than discuss work in a distant system like sand dollar or sea urchin.

There are also many other recent work from Ishihara and Mitchison which provide models for MT growth and organization in large in vitro asters from xenopus extracts which, as stated above set the state of the art for imaging and quality control, and are again omitted. Finally, the authors make incorrect statements regarding published work which are improper. To list a few: that C. elegans is not amenable to genetics, while there are tens of papers using CRISPR and RNAi in this system by leaders including Hyman/Gonzcy/Carvalho/Kimura labs that unequivocally demonstrate the requirement of dynein for sperm aster centration; or that laser ablation creates local "scars" on which to push while this method has been used by many top labs to establish the direction of MT forces; or that ciliobrevin affects MT growth, while this drug has been controlled by imaging MTs in dozens of in vitro and in vivo publications (see Cheng and Ferrel 2019 , mentioned above, for the most recent one).
Reviewer #1 (Remarks to the Author):

Comment #1.1: As the authors explain in their great introduction, the position of the astral MT network in cells, specifically its center, determines the eventual location of the spindle apparatus and ultimately the cytokinetic furrow. Positioning of the MT aster often results in its movement to the center of a cell how these forces responsible for this movement are generated and then integrated within cells of varied sizes and geometries remains an open question.

Response: We thank the review for the praise for our introduction. We have made only a few changes to this section to carve out redundant information.

Comment #1.2: This paper overall addresses this interesting question using innovative methods. The photocontrolled hydrogel system will be of interest to a broad audience of physical biologists. The major conclusion of the paper is adequately supported by the data, with the caveats below. The paper is well suited for MBoC if important revisions are made.

Response: A point-by-point response to your major and minor concerns follows. Note that we have added line numbers in the revised version to make this re-review more efficient and hopefully a bit less burdensome.

Major concerns

Comment #1.3: All the experiments are performed in egg extract where F-actin is depolymerized with a drug this is quite different from eggs. This will have a major effect on the mechanics of aster movement relative to intact eggs. This is ok, this is a standard system. But this central point must be clearly flagged for readers ideally in the abstract or intro and as a clear statement in conclusion. As it stands now, only researchers very familiar with Xenopus extract would be aware of this.

Response: We apologize for this oversight and have now made this important experimental detail more explicit. We have added the following text in lines 142-145 of the revised manuscript: “We also note that our extracts contained cytochalasin D (10 µg/ml; Desai et al., 1999), which is routinely added during preparation to disrupt the formation of filamentous actin and inhibit gelation and contraction of the extract (Field, 2014). As such, the physical properties of the extracts used here likely differ from those found in vivo.”

Comment #1.4: The experiment in Figure 4 is mis-interpreted or over-interpreted. Microtubules turn over rapidly throughout interphase asters. Therefore, the experiment reported does NOT uncouple MT growth from MTOC movement. If the authors present high resolution speckle tracking as fiducials on individual MTs they might be able to propose this interpretation. But even then, this interpretation is questionable. Fig 4 is not new information relative to previous figures and should move to a supplement.

Response: As originally written, we completely agree with the reviewer’s comment that the results depicted in Fig. 4 are either mis- or over-interpreted and that microtubules turn over rapidly in asters. In the revised manuscript, we have made extensive revisions to this section (lines 283 - 317) and would hope you will agree with our that it no longer needs to be relegated to the supplement. Our intent with these experiments was to decouple aster translocation and aster expansion, not MT growth/dynamics per se. We believe that aMTOC centration after aster MTs have grown to occupy the entirety of the
enclosure space, is an important experimental observation, largely because existing models of aster centration implicitly require that the aster moves in bulk toward cytoplasmic space that is unoccupied by astral MTs. By allowing asters to grow such that they fill the space and only then permitting the aster nucleating center to move freely, our data suggest that some degree of MT dynamicity and architectural reorganization, in terms of linkages, is also likely at play during centration. To our knowledge, this type of decoupling has not been achieved in aster centration studies though we should note that in Salle et al. 2018 aster asymmetry was induced using magnetic beads, however the asters were pulled back to the cell cortex only after centration had already occurred.

Comment #1.5: The authors state that their hydrogel is "compatible" with Xenopus extracts referencing Bisht et al., 2019. This is perhaps technically correct. However, that paper flowed extract into devices with hydrogel posts and looked at nuclear formation and import. It did not look at microtubule structures and cell cycle dynamics. More problematic is that embedding extract in a hydrogel which is done in the experiments here is very different from flowing it around posts and the majority of data in the paper is from extracts in hydrogels. It is clear in multiple figures that MT density is lowered in hydrogel experiments, perhaps unpredictably. The authors should admit about technical limitations in paragraph one of their discussion.

Response: The reviewer has correctly pointed out that the previous studies we have referenced failed to evaluate any affects that proximity to our hydrogels might have on cellular phenomenon like microtubule nucleation and growth dynamics. In the revised manuscript, we have attempted to assuage this concern by (i) referencing another recently published work from our lab in which we explicitly assessed MT dynamics in extracts surrounded by hydrogel enclosures and they resembled published rates measured in bulk extract (Geisterfer et al, Current Biology, 2020; line 140), (ii) inclusion of a new supplementary figure (Fig. S2) which shows that MT density is not affected by proximity to hydrogel barriers, (iii) we have now applied universal brightness and contrast adjustments to more accurately reflect true MT densities (we attribute the apparent differences in MT densities to issues with brightness and contrast settings erroneously applied to single images within stacks as opposed to using ImageJ’s “enhance contrast” options which are applied universally to all images within a stack) and (iv) we have expanded the second-to-last paragraph of the discussion to better address the limitations of our approach.

In regard to the specific concern about admission of technical limitations, we note that the manuscript ends with a tacit nod to the idea that our system is an in vitro system and that any observations made using this approach will need to be validated in vivo.

Comment #1.6: p150CC1 is a protein inhibitor of the dynein motor. Addition of p150CC1 to extract (Figure1B) does not affect aster centration -this is a strong experiment and would seem to favor a pushing model but it was only done in extracts while all the rest of experiments in the manuscript are performed in hydrogel. The conclusions of the paper would be better supported if one hydrogel experiment was performed with p150 CC1.

Response: Due to limitations imposed by COVID-related guidelines, we have been working at a significantly reduced experimental capacity and have been unable to perform the requested experiment at a level that merits publication (nor do we have publication quality images). However, our preliminary data suggest that aster centration
in hydrogel enclosures is largely unaffected by addition of p150-CC1. Confocal images below show an aMTOC aster centering in a ~110µm diameter hydrogel enclosure, green = EB1-GFP and red = TMBD-mCherry; note the aMTOC in the 10 min frame is not in focus as it is above the focal plane. We acknowledge that this is less than ideal, but are willing to complete these experiments in a more quantitative fashion if required.

Comment #1.7: Overall, this reviewer would like to see need to see more objective evaluation of strengths and limitations of the new methods.

Response: We appreciate the reviewer’s concern and have made numerous revisions to the text in hopes of having addressed it. The most substantial change to the manuscript in this context can be found in the discussion, specifically lines (407-437).

Minor concerns
Comment #1.8: Ref is missing from the top of page 5 aster positioning in large blastomeres (ref), aster movement away...

Response: We apologize for this oversight and have corrected this issue in the revised version of the manuscript.

Comment #1.9: Good 2018 ref mentioned in the paper is not in the reference list.

Response: Again, we apologize for this oversight and have added this reference to the reference list of the revised version of the manuscript.

Comment #1.10: I think the point of Figure 2 is asters do not center when barriers are asymmetrical, but I think the geometries are rather hard to interpret and I am not certain how they relate to pushing. Comparing a single geometry plus/minus CC1 could be more informative.

Response: Your interpretation is indeed correct and we agree that as originally presented, the relationship between the results of our studies using sawtoothed asymmetry and pushing forces is unclear. To provide more clarity on this issue, we have added a more detailed explanation of our rationale for these experiments (lines 191-199).
Comment #1.12: I think the geometry in Figure 2A makes more sense than in Figure 2B. The lobster trap is interesting but hard to interpret. With respect to pushing or pulling one could say the aster cannot go through the restricted space because the aster as a whole is too large.

Response: We agree that it is possible that aster migration through the restriction is sterically inhibited in our lobster traps and have acknowledged this alternative interpretation in the revised text (lines 357-364). These new lines of text also include several arguments as to why we believe our interpretation of the results might be more valid.

Comment #1.13: In Figure 3 I agree that faster movement away a more focused V-shaped barrier might be consistent with pushing. The less focused V-shape end up with more MT in front—if there was cytoplasmic pulling one would think the aster might move faster. However, this reviewer is not sure of the exact shape of the chamber. Are all the microtubules in front of the bead in the wide V-shape hitting a wall and slowing the aster down. I think this experiment could really benefit from p150 CC1 addition.

Response: The revised version of the manuscript includes the dimensions of the PDMS channel and we have now explicitly stated that all Vs used in our experiments were made near the center of the channel and far away from any other barriers or V’s (spacing was greater than 500 microns; lines 251-253). As for the PEGdiPDA required to repeat the V experiments in the presence of p150-CC1, its synthesis is not trivial and we have relied exclusively on our collaborator, Dr. April Kloxin, to produce it. Acquiring more has been proven extremely difficult with the current state of world affairs. See also our response to Comment #1.6.

Comment #1.14: In Figure 4, why did authors change to yet another configuration? Aster moves faster in teardrop situation—does that always happened? How do the authors interpret this?

Response: (also see response to Comment #1.4 above). We have modified the text to better explain our rational for inclusion of this geometry and set of experiments. As for why the asters seem to move faster in the teardrops versus open V’s, we admittedly do not have a good explanation for this result, however we point out that the change in speed is not statistically significant.

Reviewer #2 (Remarks to the Author):
Understanding if MTs predominantly push or pull using dynein forces to center centrosomes nuclei or spindles is an important aspect of cell division and polarity. Many initial in vitro work from Leibler/Dogterom groups used purified MTOC and MTs in small microchambers, supporting that pushing can move an aster, but the direct relevance to in vivo situations has been limited. In part because in cells, apart from few exceptions like the small fission yeast, work from many groups, in models like C. elegans, zebrafish, Xenopus or mammalian cells have long supported that centrosome centration requires dynein activity, suggesting that MT pulling from the cortex or cytoplasm is likely dominant. Here, the authors approach this problem, using a tractable Xenopus extract embedded in large ~100μm size chambers, made of PDMS or a neat photo-polymerizable hydrogel technology that even allows to change aster confinement in real-time. Their result suggests that in contrary to the established dogma in
vivo, MT pushing may be the main force that move and center asters, even if MTs are long, presumably because in large asters MTs may bundle or branch which could limit buckling. If correct, this finding could be important in the field.

Comments #2.1 & #2.2: Although I am impressed by the technology, and some of the videos, there are two major limits in the current MS, that need to be fully addressed before I could recommend publication: (i) the evidence for pushing vs pulling may be prone to caveats in the assays and interpretation; and (ii) the text is worded in a rather defensive manner omitting or mis-citing some very important literature that goes against the author’s conclusion, giving the reader the sensation of a forced message.

Response: We agree with the reviewer’s concerns on both of the issues raised here and have made every attempt to address them in our revised manuscript. This includes extensive re-writing of the text in an attempt to present the data in a more unbiased, more objective, and less defensive manner, particularly one that addresses caveats and alternative interpretations. We have also conducted a more comprehensive review of the literature and our treatment of specific references. We hope to have assuaged most of your concerns and have attempted to address more specific issues in this context in our responses below.

Comment #2.3: The main evidence supporting pushing is based on the addition of p150-CC1 glue, a potent dynein inhibitor, which does not alter aster centration in a PDMS chamber (Fig 1B). There are plausible caveats and no appropriate controls for this essential result (apart from a mitotic extract in a very different imaging set-up in supp). The use of PDMS is worrying. PDMS can rapidly deplete many proteins or drugs, and thus the authors need to directly provide a control for the lack of dynein activity while imaging centering asters in their PDMS chambers. This could be done by imaging ER/mitochondria retrograde flow as done in a recent paper by the Ferrell lab in similar extracts (Cheng, et al. Science 2019).

Response: We agree that the p150-CC1 result is critical to our main conclusion and that we had failed to adequately control for the effects of enclosing our extracts in PDMS or proximity to hydrogel structures. We have now included the requested controls in Fig. S1, which now shows that spindles can assemble normally in PDMS enclosures near hydrogel structures and that treatment with p150-CC1 indeed results in the expected phenotype even when extract is contained within a PDMS device and near hydrogel structures. We have also included the requested experiments as detailed in the Cheng & Ferrell reference and show that within PDMS devices, asters fail to collect and accumulate membranes when treated with p150-CC1 (see Fig. S1).

Comment #2.4: The authors use TMBD as a marker for MTs throughout the paper, and there is no appropriate control for it. TMBD is derived from tau which is a strong MT stabilizer. In the hands of many labs, TMBD can create an over-stabilization of MT structures, and exhibit photo-toxic effects which bundle asters or create bizarre spiral arrangements, much like those seen in Figure 1C. Thus, there is a probability that TMBD could mask the effect of adding p150-CC1 glued, or more importantly that it directly promotes MT pushing by stabilizing them.
Response: We believe that previous controlled studies from the Gatlin lab (Mooney et al., 2017) sufficiently demonstrate the appropriateness of our choice to use the mCherry-TMDB construct as a marker to visualize MTs in extracts. In that work, we demonstrated that MT dynamics were largely unaffected by addition of the probe (Fig. 3) and that the morphology of spindles assembled in extracts spiked with the construct was normal, suggesting that this fundamental cellular process, and one that is arguably particularly sensitive to changes in MT, motor, and MAP dynamics, seemed unaffected by addition of the probe.

Comment #2.5: Leaders in the field (see many recent papers from Ferrell or Mitchison labs on Xenopus based MT asters in vitro) use labelled tubulin or sir-tubulin, as better and more established probes. This defines the state of the art on which the authors should base a large set of required controls. I am willing to believe the authors' claim but I would be relatively strict with seeing a set of very clean controls, because dynein inhibition experiments are IMHO the sole support for the general claim and title of this paper.

Response: We argue that the use of our TMBD-based probe in several recent publications, some from leaders in the field, suggest that it is accepted and validated probe to visualize MT dynamics. For example, the probe has been used in recent work from the Burgess lab (Meaders and Burgess, 2020 (in revision at Cell Reports). The probe was also used to visualize microtubules in a recent publication from the Mitchison lab (Field, Pelletier, & Mitchison, Current Biology, 2020) as well as in a newish pre-print from the same lab posted to BioRxiv (Pelletier et al, 2020). We have also now tested aMTOC centration using labeled tubulin and saw no difference in p150-CC1 efficacy (Fig. S2). So although we disagree on this point, we do agree that previous work has failed to address the potential for TMBD to negatively affect the efficacy of p150-CC1 and have attempted to address this concern in the revised manuscript (see our response to Comment #2.3 above).

Comment #2.6: Indeed, the interpretation of the subsequent findings based on pushing is rather shaky. The sawtooth pattern in figure 2A may not be a very relevant support for pushing because MTs appear aligned along the long axis of the barrier, rather than pushing "end-on" onto the little negative curves. The difference in speeds in the open triangles in fig 3B and 3C could just emerge from a larger drag of the aster in 3C, as evident from the fact that they slow down as they grow, with little to do with angles of contact between MTs and chamber walls.

Response:
We agree that the interpretation of the sawtooth pattern experiments is not as straightforward as we would like. However, we argue that faster aster movement away from the sawtooth wall and the establishment of an equilibrium position further away from the sawtoothed barrier (Fig. S5), is more consistent with a pushing-based mechanism and relatively more difficult to reconcile with either pulling model.

As for the argument that the observed differences in centration speeds in our “V” experiments could be the result of increased drag as the aster grows, we argue that a cytoplasmic pulling based mechanism, the most likely alternative to pushing in this experimental setup, predicts that dynein-dependent pulling forces should also increase as a function of aster size. Indeed, the literature supports the idea that pulling forces generated by dynein-mediated transport of vesicular cargoes along aster MTs is
theoretically sufficient to overcome the drag forces (Kimura et al., 2011, Tanimoto et al., 2018) and results in a persistent constant velocity phase of aster movement that is independent of aster asymmetry (Tanimoto et al., 2018).

Comment #2.7: Finally, the stop of the aster at the lobster trap entrance, could simply result from an effective stiffness of the dense MT network around the centrosome that prevents it from moving into the trap.

Response: Reviewer #1 had the same concern. Please see our response to Comment #1.12 above.

Comment #2.8: Another important issue, is that this work contradicts recent essential literature by eminent experts in the field which are either surprisingly not cited, or mentioned so as to give the sensation that all experiments that contradict the author’s findings are incorrect! This is not proper, and the MS needs significant rewording, to address discrepancies in a more honest/transparent manner, avoiding this systematic bias. One crucial omitted paper is from Wuhr and Mitchison (Curr. Biol 2010), which clearly demonstrate the requirement of dynein using the same p150-CC1 inhibitor in Xenopus eggs in vivo. More dramatically, Cheng and Ferrel published a recent paper (Cheng Science 2019), which is also not cited, using very similar cytoplasmic interphase Xenopus extracts, which provided remarkable and very clean demonstration of the requirement of dynein activity for nuclei and centrosomes centration in large asters. How should one reconcile these findings with the one presented here? These are the Xenopus papers that the authors should confront their data with, rather than discuss work in a distant system like sand dollar or sea urchin.

Response: As mentioned above, we have made extensive revisions in order to be more transparent and less biased in terms of providing more experimental caveats, alternative interpretations of data, and remedying issues related to omitted or mis-cited references. With specific regard to this particular comment, we have now included both the Wuhr & Mitchison reference (it’s now referenced in several places throughout the text, but the most relevant new references can be found in lines 330-333 and 432-435) as well as the Cheng & Ferrell reference where appropriate (see specifically lines 293-295 and 403-406). Our omission of the Wuhr & Mitchison reference was an unfortunate mistake, as that paper was indeed focused specifically on aster positioning and in X. laevis embryos and the results of dynein inhibition via p150-CC1 were explicitly reported in supplementary Fig. 3. The omission of the Cheng and Ferrell reference was an error on our part as well. We argue, however, that the process described in that work, the self-organization of MT asters around seeded nuclei, fundamentally differs from the centration phenomenon described in our work. In contrast to centration within a cell, the nuclei and associated centrosomes in bulk extract form asters and cell-like compartments around themselves – they do not have to move from the edge of a pre-existing enclosure and then find its center. Furthermore, though qualitatively apparent, the centration of nuclei was not formally addressed or quantified in this work.

Comment #2.9: There are also many other recent work from Ishihara and Mitchison which provide models for MT growth and organization in large in vitro asters from xenopus extracts which, as stated above set the state of the art for imaging and quality control, and are again omitted.
Response: We were indeed familiar with Keisuke and Tim’s work on aster growth and our omission here was not at all done with subversive intent, we simply felt that in the context of the manuscript as it was presented, that there was no explicit need to reference their work. In hindsight, we agree that we should have, particularly in the context of aster architecture and in this revision have now included that reference (see lines 391-395) and Fig. S2, which was motivated partly by Keisuke’s work and acknowledges this in its legend.

Comment #2.10: Finally, the authors make incorrect statements regarding published work which are improper. To list a few: that C. elegans is not amenable to genetics, while there are tens of papers using CRISPR and RNAi in this system by leaders including Hyman/Gonzcy/Carvalho/Kimura labs that unequivocally demonstrate the requirement of dynein for sperm aster centration; or that laser ablation creates local "scars" on which to push while this method has been used by many top labs to establish the direction of MT forces; or that ciliobrevin affects MT growth, while this drug has been controlled by imaging MTs in dozens of in vitro and in vivo publications (see Cheng and Ferrel 2019, mentioned above, for the most recent one).

Response: We thank the reviewer for pointing out our “incorrect statements”. However, at the risk of not being properly deferential, we respectfully disagree with the implication that our work is replete with such errors. As for our first incorrect statement mentioned in the above comment, it was an obvious mistake on our part to suggest that C. elegans, arguably one of the most tractable model systems in terms of genetics, was somehow not. We meant to suggest that the other model systems routinely used to study centration were not particularly addressable in terms of genetics. We hope that the reviewer will agree that this issue has been resolved in the revised text (see lines 69-77). As for laser-induced scars, the referenced work, which describes the formation of “sniglets” specifically when chromosomes are cut by lasers, was not mis-cited, however the extension to laser ablation studies of microtubules in embryos was indeed improper as it is far too speculative. We agree with the reviewer that there is no evidence for such “scars” in the literature and have now removed any mention of such from the revised manuscript. Lastly, though we did mention that ciliobrevin affects microtubule network architecture, we did not mean to imply that the drug affects MT growth as suggested by this comment. We believe that offending statement from the previous manuscript was:

“In these same model systems, inhibition of dynein (via treatment with ciliobrevin; (Tanimoto et al., 2016; Tanimoto et al., 2018) inhibits the ability of asters to center, broadly implicating a pulling-based mechanism. We concede that it is possible that these systems do rely on dynein for aster centration, but a reasonable alternative hypothesis is that ciliobrevin, at the concentrations used, is not specific for dynein and instead inhibits a pushing based mechanism via nonspecific effects such as MT network reorganization.” The idea that ciliobrevin affects MT network reorganization comes from observations in the literature, e.g. treatment with ciliobrevin results in a major change in the pattern of microtubules formed in cycling extracts (Cheng & Ferrell) and in sea urchin embryos (personal communication from Dr. David Burgess). Though one could argue that this reorganization is a direct effect of dynein perturbation, ciliobrevin has been shown to have unexpected effects on MTs (e.g. Sainath & Gallo, 2015).
RE: Manuscript #E20-01-0088R
TITLE: "Microtubule-dependent pushing forces contribute to long-distance aster movement and centration in Xenopus laevis egg extracts"

Dear Jesse,

As you can see your manuscript has been seen by the original reviewers who both are in agreement that you have made substantial revisions to the paper, and that it is greatly improved. Reviewer 2 has two minor points that remain that you can easily address with some simple changes to the text. Thank you for sending your work to MBoC, and I will send out the "official" acceptance after you submit the new version with those minor changes.

Sincerely,
Claire Walczak
Monitoring Editor
Molecular Biology of the Cell

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Dear Dr. Gatlin,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript requires minor revisions before it can be published in Molecular Biology of the Cell, as described in the Monitoring Editor's decision letter above and the reviewer comments (if any) below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter." ) Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you have opted out of publishing the review history.

Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us immediately at mboc@ascb.org.

In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised version, and figures, please use this link (please enable cookies, or cut and paste URL): Link Not Available

Authors of Articles and Brief Communications whose manuscripts have returned for minor revision
Reviewer #1 (Remarks to the Author):

This paper introduces a new method to examine and manipulate the in vitro Xenopus extract system. It examines microtubule aster movement and finds that asters were able to find the center of artificial channels and annular cylinders, even when cytoplasmic dynein-dependent pulling mechanisms were inhibited. It is an interesting approach and expands upon the capabilities of the extract system. It is a new result and is at odds with previously published data in different systems. The modifications and additions the authors have made to this manuscript satisfy this reviewer. The main result in this manuscript is that aster movement and centering in this extract system (where F-actin is depolymerized) does not require dynein and supports a pushing model for aster movement. To inhibit dynein, authors use a dominant-negative fragment of the dynactin complex (p150-CC1) and employ the proper controls. This reagent is well validated and has been used to inhibit dynein in the extract system and eggs for years. It is similar to expression of the dominant negative p50 subunit of dynactin that is standard for dynein inhibition in tissue culture cells. Gatlin et al's result is at odds with other published experiments that used the small molecule inhibitor ciliobrevin as an inhibitor. The authors now discuss this discrepancy in the current version of manuscript (this was an important addition). Published results at odds with this manuscript are: aster movement in 100 μm diameter sea urchin eggs using ciliobrevin as an inhibitor (Tanimoto, 2016) and movement of sperm asters in 1.2 mm diameter X laevis embryos using CC1 (the latter move a bit but cannot centrate). Authors also discuss a 2019 paper by Cheng and Ferrell which purportedly demonstrates dynein inhibition by ciliobrevin affects MT aster position in a different Xenopus egg extract system. Two of these papers use ciliobrevin. One paper is uses the same inhibitor as the Gatlin lab but with somewhat different results but is in a system on a much different scale -1.2 mm egg with intact F-actin.

Ciliobrevin is a bad drug. It was discovered as an inhibitor of the dynein that supports transport in cilia and was never optimized for inhibition of cytoplasmic dynein. It does inhibit cytoplasmic dynein, but at the concentrations needed for that effect, it has non-specific side effects on microtubules that make it not useful for testing the role of dynein in microtubule organization. A case in point is the addition of ciliobrevin to extracts in the Ferrell paper that Gatlin now cites. This reviewer feels that the Ferrell paper provides evidence of the pleiotropic effects of ciliobrevin. From the chart in the
paper: compartment formation severely impaired, nuclear import severely impaired, formation of normal nuclei is largely abolished, nuclei mis-positioned, MT accumulated at border zones. Upon observation of the supplemental movie 8, this reviewer observes that MT asters form and are initially properly spaced out. Later all of the aster microtubules disappear—result of the ciliobrevin addition? Overall, addition of the drug seems to be causing a lot of problems. Non-specific effects have been known in the community for some time, but until recently were not published. This problem has finally been resolved in a paper that was just accepted for publication in Nature Reports from David Burgess's lab (this reviewer has the paper and can send a pdf is requested). In this paper, a PhD student Meaders, found a discrepancy between effects of ciliobrevin and CC1 in their ability to block pronuclear migration in sea urchin eggs. They showed that ciliobrevin causes non-specific inhibition of microtubule. Their results in sea urchin support the Gatlin results here.

This reviewer realizes it is perhaps not appropriate to bring up data that is in press. As I mentioned above, I can provide the paper if requested. This reviewer does this in an effort to point out why there could be differences between results.

If we can agreed that CC1 is a valid dynein inhibitor, we can consider the rest of the paper. The paper has been rewritten, experiments better explained and multiple references added and discussed. Gatlin et al provide convincing evidence that asters are pushed to the center by microtubule growth in their system. This is a new and interesting finding for a large-cell system. This is an interesting step forward in the cell biology of large cell systems and merits publication in MBC. The publication of this paper (and the Burgess paper) will hopefully stimulate experiments and discussion in the field to clarify the roles of both inhibitors.

Reviewer #2 (Remarks to the Author):

The revised MS by Sulerud et al. is much improved. Especially the new data contained in Fig S1 were critical to support author's claim, and make a convincing case for the role of pushing forces for centering even up to 100 micron size spaces. The writing is also improved with regard to the rest of the litterature and the limits of the results presented here. The last paper by the Mitchison lab on aster motion in vitro (Pelletier et al. BioRxiv 2020) should also be cited and discussed. It is another experiment where addition of CC1 in extracts with fragmented actin halts large aster motion immediately. This is a very different result than in here. Please cite and comment.

I still stand that the interpretation of the Lobster trap experiment is not as simple as the authors write. line 218: "In the absence of other types of forces, the net centering force ...". As said in my first rebuttal, there is an additional elastic force from the local stiffness of the aster around the aster center that pushes back the aster, as it tries to move within the trap. Thus the argument used in the text is not correct. This section needs some re-writting.
September 16th, 2020

Dear Dr. Walczak,

This cover letter accompanies our revised manuscript titled “Microtubule-dependent pushing forces contribute to long-distance aster movement and centration in Xenopus laevis egg extracts”. We have attempted to address the two remaining concerns of reviewer #2 by rewording the appropriate sections of text. Specifically, we have now better described the rationale for our interpretation of the included “lobster trap” experiments and have more explicitly acknowledged that the alternative interpretation presented by reviewer #2 is not ruled out by our approach. In addressing this concern, we have now referenced the BioRxiv manuscript from James Pelletier and the Mitchison lab. We have also cited the in press manuscript that Reviewer #1 mentioned where appropriate.

Thank you so much for your time and effort in shepherding this work through the editorial process and we look forward to seeing it published in MBoC! Please contact me if you require any additional information or action on our part.

With best regards,

Jesse “Jay” Gatlin
Associate Professor of Molecular Biology
RE: Manuscript #E20-01-0088RR
TITLE: "Microtubule-dependent pushing forces contribute to long-distance aster movement and centration in Xenopus laevis egg extracts"

Dear Jesse

Thank you for making the changes as requested by the reviewers. I am happy to accept your paper for publication in Molecular Biology of the Cell. Thank you for sending your work to our society journal and supporting great cell biology.

Sincerely,
Claire Walczak
Monitoring Editor
Molecular Biology of the Cell

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Dear Dr. Gatlin:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

Within approximately four weeks you will receive a PDF page proof of your article.

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We are pleased that you chose to publish your work in MBoC.

Sincerely,

Eric Baker
Journal Production Manager
