EMBO reports

Two-step nuclear centring by competing microtubule- and actin-based mechanisms in 2-cell embryos

Yunan Ye and Hayden Homer
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(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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision 23rd May 2022

Dear Dr. Ye

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

As you will see, the referee's opinions on the significance of your findings are divided. We have discussed these reports within the team and we appreciate that your study is the first to describe nuclei positioning after the first mitotic division in mouse embryos, the interest of which is acknowledged by both, referee 1 and 2. On balance, we have decided to give you the chance to revise your study for potential publication in EMBO Reports. Please address all concerns raised by the referees and provide all requested control experiments to strengthen your data. 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 (August 23, 2022). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

You can either publish the study as a short report or as a full article. For short reports, the revised manuscript should not exceed 27,000 characters (including spaces but excluding materials & methods and references) and 5 main plus 5 expanded view figures. The results and discussion sections must further be combined, which will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. For a normal article there are no length limitations, but it should have more than 5 main figures and the results and discussion sections must be separate. In both cases, the entire materials and methods must be included in the main manuscript file.

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:

1) A data availability section providing access to data deposited in public databases is missing. If you have not deposited any data, please add a sentence to the data availability section that explains that.

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.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

When submitting your revised manuscript, we will require:

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

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.

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) 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.

9) 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 .

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

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.

Yours sincerely,

Martina Rembold, PhD
Senior Editor
EMBO reports

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Referee #1:

The work by Ye and Homer deciphers the mechanisms of nucleus positioning in 2-cell embryos. They present challenging but generally convincing experiments which shows that F-actin and microtubules have antagonistic roles in this process: microtubules favoring off-centering of the nucleus at the beginning of the 2-cell stage while F-actin promoting the centering in a second step. This is a nice piece of work that explores a stage not studied before and which is well-supported by the data presented. Globally, it is not extremely original, nor very novel conceptually in terms of mechanism, yet the description of this process is important for the field of early mammalian embryogenesis since improper nuclear positioning will lead to asymmetric blastomere formation. I only have minor comments on the work.

1/ It would be nice if authors could document better actin organization in two-cell embryos in controls and after treatments with SMIFH2, CytoD, etc... Indeed, the data presented in Figure 5E and F could be improved both in terms of resolution and in terms of cytoplasmic actin mesh description.

2/ In Figure 2, I believe authors could change the reference time to render the data clearer. They present two types of challenging experiments where drugs were added before or after nuclei off-centering, yet the data do not show this explicitly (B-H). It would be nice to grasp immediately from the series of the images (B-F) and graphs (G and H) that the time of reference is different for B/C versus D/F and for G versus H.

3/Authors show that off-centered nuclei, in the absence of microfilaments (Figure S2B), remain off-centered and do not drift from their initial position. This could suggest, as previously shown by Almonacid NCB 2015 on nuclear oocyte centering and by Chaigne Nat Comm 2016 on pronuclear centering in the one-cell zygote, that viscosity is high in two-cell embryos. Could author test this hypothesis further? Could they directly measure viscosity?

4/ Previous work done on mouse oocytes has already shown antagonistic action from F-actin and microtubules on nuclear centering (Almonacid NCB 2015; Almonacid Dev Cell 2019). These two works could be discussed together with the data on the two-cell stage presented here.

Referee #2:

Ye and Homer investigated the mechanism of nucleus positioning during early mitotic division in mouse embryos. The authors found two different phases of nucleus positioning following first mitotic anaphase: a) the movement of the newly formed nucleus towards the cortex (center-to-cortex travel) and b) the centering of the off-center nucleus. Using time-lapse confocal imaging and chemical manipulations, the authors found that center-to-cortex movement is microtubule dependent, whereas nucleus centration is actin dependent. Center-to-cortex nucleus movement seems to be dispensable for subsequent embryonic development.

The manuscript is well written, and it is the first to study nucleus positioning after the first mitotic division in mouse embryos. However, my main concern is that the significance of the findings is not clear. Also, there are several over-interpretations throughout the manuscript. The authors need to tone down several statements and discuss the limitation of this study carefully.

1- Lines 122-123: The authors mentioned "nuclei did not return to the cell-centre along the initial centre-to-cortex path thereby resulting in an overall loop-shaped journey". In Movie S1, it seems that the embryo itself undergoes a minimal rotation which may be the cause of this nuclei "loop-shaped journey". The authors should take embryo movement into consideration.
2- Lines 219-225: After cytochalasin D washout, 21 out of 23 blastomeres showed nuclei centration, whereas the remaining two blastomeres had nuclei that remained off-center. Because only one of these two blastomeres assembled an off-center spindle leading to asymmetrical division, the authors concluded "post-NEBD events may not be able to re-position the spindle before the subsequent division". Given the occurrence of this phenotype in only one blastomere, this conclusion is too strong to be mentioned.
3- Fig. 4A: This experiment lacks control group and quantifications with the proper statistical analysis.
4- The authors found abnormal nucleus movement upon treating the zygotes with Brefeldin A. It is unclear how Brefeldin A results in chaotic nucleus positioning especially this phenotype is different from that of cytochalasin D (disrupting F-actin).
5- The authors used MyoVin-1 inhibitor to inhibit Myosin-Vb in oocytes and 2-cell embryos. The authors found no effect of MyoVin-1 on nucleus positioning in embryos. How did the authors confirm the efficiency of this compound concentration to inhibit Myosin-V in mouse embryos?
6- The finding (Fig. 5C,D) that only nuclei, but not oil droplets, can move to the center is interesting and showed a clear difference from oocytes. However, the number of examined embryos is too low (n=9). How many replicates?
7- The authors examined the role of cortical actin on nucleus centration by inhibiting formins (using SMIFH2, please note that SMIFH2 is not formin specific) and Arp2/3 (using CK666). Unlike Arp2/3, formins can regulate F-actin nucleation at both the cortex and the cytoplasm. First, please confirm the effect of SMIFH2 on cytoplasmic and cortical F-actin. The authors found loss of nucleus centration upon treating the embryos with SMIFH2 but not CK666. This finding suggests that cytoplasmic actin may play a role and, therefore, the conclusion that nucleus centration depends only on cortical F-actin needs to be revised.

8- To investigate the role of dynein in nucleus movement to the cortex, the authors used dynarrestin to inhibit dynein and found that dynarrestin does not affect such movement. The authors should confirm the efficiency of dynarrestin (at the selected concentration) to inhibit dynein in mouse embryos.

Referee #3:

This is a straight-forward paper that examines how nuclei become centered in mouse 2 cell blastomeres immediately after the first cleavage. It is largely descriptive and shows that nuclei continue to migrate to the cell cortex following the first cleavage and so have to be recentered. Migration to the cortex is demonstrated to be MT dependent and re-centering requires formin-nucleated actin and is independent of MTs. The authors contrast this to the highly asymmetric events in meiosis and the centering of pronuclei in the zygote. Centering of the male pronucleus also requires actin but MTs also play some role. The authors discuss that the absence of centrosomes may make this cell type more dependent upon the actin cytoskeleton. Although they should be more accurate as the mouse embryo is more accurately missing centrioles at this stage and has MTOCs that provide centrosomal function. One point that the authors fail to either discuss or investigate is that the actin cytoskeleton has become drastically rearranged in the cells they are studying as a result of the very first symmetrical cytokinesis.

In summary, these experiments have been carefully executed and should be published. However, there is nothing particularly startling here and the findings are of insufficient general interest for EMBO Reports. My feeling it that they would find a better home in a journal devoted to development.

We would like to thank the reviewers for taking the time to review our manuscript, provide insightful comments and constructive criticisms. We have undertaken a number of new experiments, and hope that the reviewers find our conclusions sufficiently strengthened.

Importantly, we have included new experiments involving live-cell particle-tracking microrheology, which enabled us to measure the cytoplasmic viscosity of 2-cell mouse embryos for the first time thereby broadening the scope and interest of the paper. These results surprisingly revealed that the cytoplasmic viscosity of 2-cell embryos is over 7 times greater than mouse oocytes perhaps explaining why diffusive mechanisms used by oocytes for nuclear positioning do not suffice in embryos for nuclear centring without additional actin-driven forces.

We have also undertaken higher-resolution imaging of actin networks in embryos and provide detailed quantifications of cytoplasmic and cortical actin intensities to address questions regarding the relative contribution of each actin population to nuclear centring.

In the revised manuscript, text changes are shown in red. Changes to the figures are as follows:

- Figure 1 (no change)
- Figure 2 (modified representation)
- Figure 3 (no change)
- Figure 4 (modified A, C)
- Figure 5 (new D - I)
- Figure 6 (new figure)
- Figure 7 (new figure modified from Fig. S5)
- Figure S1 (new C)
- Figure S2 (no change)
- Figure S3 (no change)
- Figure S4 (new figure)

Below we present a point-by-point response to Reviewers' questions.

Referee #1:

The work by Ye and Homer deciphers the mechanisms of nucleus positioning in 2-cell embryos. They present challenging but generally convincing experiments which shows that F-actin and microtubules have antagonistic roles in this process: microtubules favoring off-centering of the nucleus at the beginning of the 2-cell stage while F-actin promoting the centering in a second step. This is a nice piece of work that explores a stage not studied before and which is well-supported by the data

presented. Globally, it is not extremely original, nor very novel conceptually in terms of mechanism, yet the description of this process is important for the field of early mammalian embryogenesis since improper nuclear positioning will lead to asymmetric blastomere formation. I only have minor comments on the work.

1/ It would be nice if authors could document better actin organization in two-cell embryos in controls and after treatments with SMIFH2, CytoD, etc... Indeed, the data presented in Figure 5E and F could be improved both in terms of resolution and in terms of cytoplasmic actin mesh description.

Authors' response:

We thank the reviewer for this suggestion and have performed multiple additional live and fixed experiments to address these concerns.

1. Experiments in Figure 5E, F in the original manuscript were repeated using a x63 objective to produce images with improved resolution, which are presented in the new Figure 6. We found cortical actin enrichment consistently occurred upon nuclear approach (Figure 6A). We also undertook new experiments using higher temporal resolution by imaging at 3 min-intervals rather than 15 min intervals during the window when nuclei approached the cortex and cytochalasin D was added. As before, we found that cytochalasin D rapidly (within 5-10 min) disrupted cortical actin overlying approaching nuclei and that nuclei then stagnated at the cortex (Figure 6B in revised manuscript).
2. For analysing actin further, we undertook new analyses of fixed embryos immunostained for phalloidin following treatment with DMSO, cytochalasin D, latrunculin, SMIFH2, jasplakinolide and blebbistatin. High-resolution images were captured using the x63 objective and the intensities of both cortical and cytoplasmic actin were quantified (Appendix Fig S4 E, F of the revised manuscript) thereby providing a more comprehensive analysis of both cytoplasmic and cortical actin as requested by the reviewer. We found a clear association between nuclear movement and cortical actin but not with cytoplasmic actin. We acknowledge, however, that actin-related inhibitors may have differing effects on multiple actomyosin assemblies and have added a discussion paragraph on the possible contributions of both cortical and cytoplasmic actin in the revised manuscript (line 514 - 525).

2/ In Figure 2, I believe authors could change the reference time to render the data clearer. They present two types of challenging experiments where drugs were added before or after nuclei off-centering, yet the data do not show this explicitly (B-H). It would be nice to grasp immediately from the series of the images (B-F) and graphs (G and H) that the time of reference is different for B/C versus D/F and for G versus H.

Authors' response:

We thank the reviewer for raising this very important point. We have modified Figure 2 to make the timing of drug addition clearer.

3/Authors show that off-centered nuclei, in the absence of microfilaments (Figure S2B), remain off-centered and do not drift from their initial position. This could suggest, as previously shown by Almonacid NCB 2015 on nuclear oocyte centering and by Chaigne Nat Comm 2016 on pronuclear centering in the one-cell zygote, that viscosity is high in two-cell embryos. Could author test this hypothesis further? Could they directly measure viscosity?

Authors' response:

We thank the reviewer for this suggestion, which motivated us to improve the depth and scope of our paper. To directly probe the mechanical properties of the cytoplasm without contacting the cell surface, we performed intracellular particle tracking microrheology, which has been used in early embryos of *C.elegans* and *Drosophila*, and fibroblasts (Daniels et al., 2006, Kole et al., 2005, Wessel et al., 2015) and reviewed by (Wirtz, 2009) but not yet in mouse oocytes and embryos.

Briefly, the cytoplasmic viscosity of embryos can be determined from the microscopic motion of spheric particles embedded in the cytoplasm (Mason, 2000). Thus, we chose 1 µm-diameter passive carboxylate-modified microsphere beads, a size similar to endogenous actin vesicles (Almonacid et al., 2015).

As the reviewer correctly predicted, cytoplasmic viscosity in 2-cell embryos is very high. Reported viscosity using similar intracellular techniques ranged from 0.67 to 1 Pa.S in *C. elegans* embryos (Khatri et al., 2022, Daniels et al., 2006), and was around 1 Pa.S in *Drosophila* early embryos and serum-starved fibroblasts (Kole et al., 2004, Wessel et al., 2015). Similarly, our analyses of mouse oocytes found that their viscosity was 0.48 Pa.S. Strikingly, however, we found the viscosity of 2-cell embryos was over 7 times higher than mouse oocytes, reaching 3.61 Pa.S (new Figure 5 in the revised manuscript). Note that we do not compare our viscosity values with those obtained from active microrheology (e.g. Magnetic tweezers) as they measure non-equilibrium properties, or with those derived from extracellular approaches (e.g. Micropipette suction) as they also probe membrane mechanics. Increased cytoplasmic viscosity could help explain why embryos require additional nuclear centring mechanisms that produce more force than active diffusion.

4/ Previous work done on mouse oocytes has already shown antagonistic action from F-actin and microtubules on nuclear centering (Almonacid NCB 2015; Almonacid Dev Cell 2019). These two works could be discussed together with the data on the two-cell stage presented here.

Authors' response:

We thank the reviewer for pointing this out. We have incorporated this in the revised Discussion (lines 491 - 494).

Referee #2:

Ye and Homer investigated the mechanism of nucleus positioning during early mitotic division in mouse embryos. The authors found two different phases of nucleus positioning following first mitotic anaphase: a) the movement of the newly formed nucleus towards the cortex (center-to-cortex travel) and b) the centering of the off-center nucleus. Using time-lapse confocal imaging and chemical manipulations, the authors found that center-to-cortex movement is microtubule dependent, whereas nucleus centration is actin dependent. Center-to-cortex nucleus movement seems to be dispensable for subsequent embryonic development.

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1- Lines 122-123: The authors mentioned "nuclei did not return to the cell-centre along the initial centre-to-cortex path thereby resulting in an overall loop-shaped journey". In Movie S1, it seems that the embryo itself undergoes a minimal rotation which may be the cause of this nuclei "loop-shaped journey". The authors should take embryo movement into consideration.

Authors response:

We thank the reviewer for this very interesting observation. We agree entirely that at least part of the apparent loop-shaped journey may be accounted for by embryo rotation. We have therefore changed the wording accordingly in the revised manuscript (lines 118 - 120 in the revised manuscript) and include images that illustrate embryo rotation occurring concurrently with nuclear movement (Appendix Figure S1C in the revised manuscript).

2- Lines 219-225: After cytochalasin D washout, 21 out of 23 blastomeres showed nuclei centration, whereas the remaining two blastomeres had nuclei that remained off-center. Because only one of these two blastomeres assembled an off-center spindle leading to asymmetrical division, the authors concluded "post-NEBD events may not be able to re-position the spindle before the subsequent division". Given the

occurrence of this phenotype in only one blastomere, this conclusion is too strong to be mentioned.

Authors' response:
We agree with the Reviewer and have deleted this section.

3- Fig. 4A: This experiment lacks control group and quantifications with the proper statistical analysis.

Authors' response:
We thank the reviewer for pointing this out and have now included experiments with a DMSO-treated control group (Figure 4A in the revised manuscript). We quantified nuclear position at 5h post-anaphase in DMSO- and BFA-treated groups, by which time untreated control embryos have re-centred their nuclei (see Fig. 1A, C). Consistent with impaired nuclear centring following BFA treatment, we find that BFA-treated embryos exhibit significantly higher d values by 5 h post-anaphase compared with DMSO-treated controls (Figure 4A, C in the revised manuscript).

4- The authors found abnormal nucleus movement upon treating the zygotes with Brefeldin A. It is unclear how Brefeldin A results in chaotic nucleus positioning especially this phenotype is different from that of cytochalasin D (disrupting F-actin).

Authors' response:
The reviewer is correct that BFA resulted in a different phenotype from cytochalasin D. We would like to emphasise that BFA was used primarily as a tool for enabling us to assess the consequence of off-centred nuclei on the subsequent division without blocking cytokinesis as occurs with actin inhibitors. We have, however, performed additional experiments to address how BFA affects nuclear positioning.

Based on the distorted nuclear shapes observed following BFA treatment, we hypothesised that BFA results in abnormal forces that act at the nuclear surface and that these forces could be the consequence of aberrant structures in the cytoplasm. Since a well-known effect of BFA is to disrupt golgi and ER organisation, and to induce a so-called BFA compartment (Nebenfuhr et al., 2002), we used diI to label membranous structures. We found that in the presence of DMSO, diI exhibits uniform distribution throughout 2-cell embryos. In contrast, following BFA treatment, diI staining becomes much less uniform with hyper-intense signals often located between nuclei and the cell-centre (see Figures below A, B)[Figures for referees not shown.] . This could represent aberrant golgi/ER structures, which may physically interfere with nuclear centring. A similar mechanism was previously proposed by (Scheffler et al., 2021).

We have not included this data in the revised manuscript as our intention was to use BFA as a tool for decentring nuclei without severely compromising cytokinesis rather than to decipher mechanisms of BFA action. We would, however, be happy to follow the advice of the journal.

5- The authors used MyoVin-1 inhibitor to inhibit Myosin-Vb in oocytes and 2-cell embryos. The authors found no effect of MyoVin-1 on nucleus positioning in embryos. How did the authors confirm the efficiency of this compound concentration to inhibit Myosin-V in mouse embryos?

Authors' response:

MyoVin-1 was used at 30 μM in neuronal cell cultures (Gramlich and Klyachko, 2017, Maschi et al., 2021). We found that at this concentration, MyoVin-1 disrupted nuclear positioning in oocytes (Figure 5C in revised manuscript), entirely consistent with a known requirement for Myosin Vb in oocyte nuclear positioning (Almonacid et al., 2015) thereby confirming the efficacy of this dose. Notably, however, the same concentration (30 μM) did not affect nuclear positioning in 2-cell embryos (Figure 5C in revised manuscript). We also tried a higher dose (50 μM) and obtained the same results.

It is worth noting that we have now strengthened the argument that oocytes and embryos use different nuclear positioning mechanisms with a new set of experiments showing that 2-cell embryos have a markedly higher intracellular viscosity than oocytes (Fig. 5E – I in revised manuscript), which we detail further in our responses to Reviewer 1.

6- The finding (Fig. 5C,D) that only nuclei, but not oil droplets, can move to the center is interesting and showed a clear difference from oocytes. However, the number of examined embryos is too low (n=9). How many replicates?

Authors' response:
We acknowledge the reviewer's concerns. We have now undertaken an additional replicate experiment bringing the total number of replicates to 4 resulting in a total number of analysed embryos of n = 14. The results calculated with the inclusion of these additional 5 embryos are entirely consistent with our previous data (Figure 5B in the revised manuscript). While we injected more embryos, only oil droplets that positioned near the cortex along with the nuclei were suitable for analysis.

7- The authors examined the role of cortical actin on nucleus centration by inhibiting formins (using SMIFH2, please note that SMIFH2 is not formin specific) and Arp2/3 (using CK666). Unlike Arp2/3, formins can regulate F-actin nucleation at both the cortex and the cytoplasm. First, please confirm the effect of SMIFH2 on cytoplasmic and cortical F-actin. The authors found loss of nucleus centration upon treating the embryos with SMIFH2 but not CK666. This finding suggests that cytoplasmic actin may play a role and, therefore, the conclusion that nucleus centration depends only on cortical F-actin needs to be revised.

Authors' response:
We thank the reviewer for raising this important point and acknowledge that actin-related inhibitors may have effects on different populations of actomyosin assemblies.

The reviewer asks about "the effect of SMIFH2 on cytoplasmic and cortical actin". In response, we undertook new experiments in both live and fixed oocytes.

1. In the first set of new experiments, F-actin was analysed in live oocytes using UtrCH-mCherry (Fig. 6D in the revised manuscript). Following SMIFH2 addition, we observed a rapid disappearance of cortical actin enrichment overlying nuclei (Figure 6D). This was followed by a global increase in cortical and cytoplasmic F-actin that may reflect homeostatic F-actin assembly driven by other competing nucleators (Burke et al., 2014).
2. In the second set of new experiments, we analysed actin in embryos that were fixed and immunostained for phalloidin after either SMIFH2 or DMSO addition. High-resolution images were captured using the x63 objective and the intensities of both cortical and cytoplasmic actin were quantified (Appendix Fig S4 E, F of the revised manuscript). Analysis of cortical actin intensity confirmed the reduction we observed during live imaging shortly after SMIFH2 addition whereas we found no change in cytoplasmic actin at this stage. We undertook additional new analyses of cytoplasmic and cortical actin levels following treatment with cytochalasin D, latrunculin, jasplakinolide and blebbistatin (Appendix Fig S4 E, F of the revised manuscript). In all cases, we

found a clear association between nuclear movement and cortical actin but not with cytoplasmic actin, strongly suggesting that it is cortical rather than cytoplasmic actin that is the major driver for nuclear re-centring. We acknowledge, however, that cytoplasmic actin networks could still be involved in nuclear positioning and have added a paragraph discussing this and the limitations of using actin-related inhibitors (line 514 - 525).

8- To investigate the role of dynein in nucleus movement to the cortex, the authors used dynarrestin to inhibit dynein and found that dynarrestin does not affect such movement. The authors should confirm the efficiency of dynarrestin (at the selected concentration) to inhibit dynein in mouse embryos.

Authors' response:
Dynein inhibition is known to compromise spindle pole focusing leading to gross defects in spindle bipolarity and structure, e.g. (Van Heesbeen et al., 2014). In keeping with effective dynein inhibition at the concentration of dynarrestin used in our experiments, we observed abnormal spindle structure during live imaging of dynarrestin-treated embryos (Fig. 7D of revised manuscript, 21.5 – 23.5h). We undertook new analyses to quantify the proportion of bipolar spindles with equatorially aligned chromosomes in DMSO- and dynarrestin-treated embryos and find that the latter completely fail to assemble canonical bipolar spindles (Fig. 7F in the revised manuscript) strongly supporting that the dose used was inhibitory to dynein.

Referee #3:

This is a straight-forward paper that examines how nuclei become centered in mouse 2 cell blastomeres immediately after the first cleavage. It is largely descriptive and shows that nuclei continue to migrate to the cell cortex following the first cleavage and so have to be recentered. Migration to the cortex is demonstrated to be MT dependent and re-centering requires formin-nucleated actin and is independent of MTs. The authors contrast this to the highly asymmetric events in meiosis and the centering of pronuclei in the zygote. Centering of the male pronucleus also requires actin but MTs also play some role. The authors discuss that the absence of centrosomes may make this cell type more dependent upon the actin cytoskeleton. Although they should be more accurate as the mouse embryo is more accurately missing centrioles at this stage and has MTOCs that provide centrosomal function. One point that the authors fail to either discuss or investigate is that the actin cytoskeleton has become drastically rearranged in the cells they are studying as a result of the very first symmetrical cytokinesis.

In summary, these experiments have been carefully executed and should be published. However, there is nothing particularly startling here and the findings are of insufficient general interest for EMBO Reports. My feeling it that they would find a better home in a journal devoted to development.

Authors' response:
We thank the reviewer for the feedback.

In response to the reviewer's comment regarding centrosomes, we have revised the Discussion sentence "Early mitotic divisions in mouse embryos occur without centrosomes" (lines 469 - 470), and included the qualifier "canonical centriole-containing" before "centrosomes".

The reviewer raises an insightful point regarding "drastically rearranged" changes following the "first symmetrical cytokinesis". We have broadened the scope of our findings in the revised manuscript with a new set of challenging experiments directly measuring intracellular viscosity in 2-cell embryos and oocytes with particle-tracking microrheology. This technique has been used in early embryos of *C.elegans* and *Drosophila*, and fibroblasts (Daniels et al., 2006, Kole et al., 2005, Wessel et al., 2015), reviewed by (Wirtz, 2009), but not previously in mouse oocytes or embryos. We found that cytoplasmic viscosity in embryos is over 7 times higher than in oocytes (Figure 5E – I in the revised manuscript), which could explain why 2-cell embryos require additional force-producing mechanisms beyond diffusion-mediated ones to centre nuclei whereas diffusion-based mechanisms appear to suffice in oocytes. This is the first dataset of passive, intracellular microrheology of mouse oocytes and embryos. These data support the reviewer's suggestion that changes arising during symmetrical divisions result in different properties, and unique challenges, in embryos compared with oocytes.

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1st Revision - Editorial Decision 1st Sep 2022

Dear Ms. Ye

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

As you will see, both referees are very positive about the study and support publication in EMBO reports.

Browsing through the manuscript myself, I noticed a few editorial things that we need before we can proceed with the official acceptance of your study.

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- Please include all information on funding in the Acknowledgement section.

- Appendix: please list each figure separately in the table of content and include page numbers

- Appendix Figure S2: Please only define the p-values actually shown in panel B (*, **)

- Appendix Figure S4: Correct "In (A- D), scale bar = ..." to "In (A-C) as D shows no scale bar.

- Author Checklist: Please complete the section Ethics - Studies involving experimental animals - authority granting ethics approval.

- Figure callouts:
- Fig 2 panel callouts are not alphabetical. If possible, rearrange this figure so that the panels can be called out in an alphabetical manner.
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- Please double-check the composition of panel 5E, overview and zoomed region and please provide the source data for this image.

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

With kind regards,

Martina Rembold, PhD
Senior Editor
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Referee #1:

Authors have performed all the experiments and slight changes I suggested, which I think give more strength to the revised manuscript, hence I am fully satisfied by their revision.

Referee #2:

The authors have addressed all my major concerns. I am satisfied with the revision and suggest its publication.

The authors have addressed all minor editorial requests.

2nd Revision - Editorial Decision 12th Sep 2022

Ms. Yunan Ye
University of Queensland
Building 71/918, RBWH Campus
Herston
Brisbane, Queensland 4029
Australia

Dear Ms. Ye,

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

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The data shown in figures should satisfy the following conditions:
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