

Microtubule pushing and cortical pulling cooperate to orient and position the mitotic spindle

Alikhan Yeltokov, Tara Finegan, Alexander Fletcher, Holly Lovegrove, and Dan Bergstralh

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Review Timeline:

Submission Date:	20th Mar 26
Editorial Decision:	22nd May 26
Revision Received:	28th Jul 26
Editorial Decision:	9th Sep 26
Revision Received:	16th Sep 26
Accepted:	20th Sep 26

Editor: Ieva Gailite

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. 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.)

Dear Dr. Bergstralh,

Thank you for submitting your manuscript to The EMBO Journal. We have now received reports from three reviewers, which I have included below.

As you can see, reviewers #1 and #2 are more appreciative of the findings, while also indicating a number of concerns about the presented model that would need to be addressed before they can support publication of the study. On the other hand, reviewer #3 is critical on the contribution of the proposed model of spindle orientation regulation due to its 2D nature and the omitted contribution of pulling forces.

Due to the substantive nature of these concerns, especially those raised by reviewers #1 and #3, I would appreciate if you could let us know to what extent you would be able or willing to address the concerns of all reviewers during a revision. Such a brief preliminary point-by-point response would be very helpful for the final editorial decision.

Please let me know if you have any questions about this pre-decision consultation approach. I look forward to discussing the potential revision with you.

With best wishes,

Ieva

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Referee #1 (Report for Author)

Summary

This manuscript presents a generalizable 2D computational model incorporating both cortical microtubule (MT) pulling and polymerization-based pushing forces to investigate mitotic spindle orientation and positioning across diverse cell types. The model is validated against multiple experimental systems, such as *Drosophila* follicular epithelial cells, neuroblasts, and the *C. elegans* embryo, before being applied to the more challenging case of irregularly shaped zebrafish intersegmental vessel (ISV) endothelial cells. The authors conclude that pulling forces are the primary determinants of directed spindle orientation, while pushing forces play essential supporting roles by preventing spindle stalling, promoting dynamicity, and, in the zebrafish context, encoding cell geometry to drive spindle orientation independently of pulling. The paper is clearly written, the model is thoughtfully constructed, and the biological scope is impressive. However, several points require clarification or additional justification.

Major Comments

1. Missing citation for laser ablation evidence (Line 41)

The claim at line 41 that laser ablation of astral MTs triggers spindle recoil is supported by citations to Bosveld et al. (2016) and Wu et al. (2024). However, the foundational laser ablation experiments demonstrating pulling forces on centrosomes by Stephan Grill and colleagues (Grill et al., 2001, *Nature*, and related work) should be cited here, as this is the primary evidence for cortical pulling forces in the field. The current citation list gives the impression this is a recent discovery rather than a well-established result.

2. Figure 1D and 1E: Dynamic instability of MTs not represented (Figure 1D, 1E)

In Figure 1D, the pushing MT is depicted as a static filament, with no indication that it is in a growing (polymerizing) state. This is biologically important because pushing forces arise specifically from growing MT plus-ends encountering the cortex. A shrinking MT would not generate a pushing force. The authors explicitly state in the text (line 140-141) that pushing occurs when a "growing MT reaches the cortex," yet the figure does not convey this. The same issue applies to Figure 1E, the schematic should distinguish growing (pushing) from shrinking MTs more clearly in the context of force generation, consistent with the color-coding introduced later in Figure 2B. The authors should either add a polymerization indicator (e.g., growing tip markers) or an explicit label to clarify that this depicts a growing MT.

3. Model parameters: MT and force generator numbers require clearer justification

The authors use astral MT numbers scaled from *C. elegans* estimates (500-1000 per aster; Kozlowski et al., 2007) and a range of 40-400 force generators (FGs) citing Grill et al. (2003). The manuscript justifies its FG range of 40-400 citing Grill et al. (2003) for the estimate that ~50 FGs are required for spindle positioning. Pécréaux et al. (2006) uses $N = 28$ motors per side of the AP axis (~56 total) in their model of anaphase oscillations in *C. elegans*, which is broadly consistent with the lower end of the range. However, both estimates come from anaphase dynamics in *C. elegans* specifically. The authors should clarify whether these numbers are appropriate starting points for the smaller *Drosophila* cells, where the range of 40-400 spans an order of magnitude without clear biological justification for the upper bound.

4. The spatial distribution of motors/FGs on the cortex is a critical parameter that shapes the outcome but is not systematically discussed. How sensitive are the results to the assumed spatial arrangement of FGs, particularly in the neuroblast and follicle cell conditions? The FG distribution is derived from fluorescence intensity profiles (Mud signal), but the mapping from fluorescence intensity to motor number is not validated. A sensitivity analysis varying FG distribution shape (e.g., width and peak position of the gradient) would substantially strengthen the modeling conclusions.

5. For *Drosophila* cells, the MT numbers used in simulations (50-400 in Figure 2D) are substantially below the *C. elegans* scaling estimate. The justification for this range in *Drosophila* specifically should be stated.

6. Tension with Pécréaux et al. (2016) on the role of pushing vs. pulling during *C. elegans* metaphase (Figure 3B)

Pécréaux et al. (2016) directly addresses spindle centering during metaphase in the *C. elegans* one-cell embryo. That paper independently estimates that ~1000 active force-generating elements underlie metaphase centering, which is consistent with the MT numbers used by Yeltokov et al. (500-1000 per aster from Kozlowski et al. 2007). However, Pécréaux et al. (2016) reach a mechanistically important conclusion, they propose that the high precision of metaphase centering is accounted for quantitatively by MT pushing alone, and cortical pulling forces are argued to become dominant only during anaphase. This creates a direct tension with the Yeltokov model, which simultaneously incorporates both cortical pulling and pushing forces during the phase modelled in Figure 3B.

This tension is not acknowledged or discussed anywhere in the manuscript. The authors cite Pécréaux et al. (2016) only for simulation duration parameters (line 151), while overlooking its mechanistic conclusions that are directly relevant to their modeling choices. They should address this contradiction. Without this discussion, the paper's claim

to provide a "unified framework" for *C. elegans* spindle dynamics is incomplete.

7. Experimental validation of threshold behavior (Lines 216-225)

The authors attempt to validate the model's predicted threshold behavior by depleting Mud using Traffic jam-GAL4 / UAS-mud-shRNA at varying temperatures (25°C, 27°C, 29°C). While the results are consistent with the model prediction, there is a significant confound, temperature affects not only GAL4 activity (and thus Mud levels / FG number) but also MT dynamics, including polymerization rate, catastrophe frequency, and rescue rate. This means the experiment is not cleanly changing a single parameter. The authors acknowledge none of this. At minimum, they should discuss this limitation explicitly and note that the temperature-dependent effects on MT dynamics could independently alter spindle behavior. Ideally, they would either cite evidence that MT dynamics are not significantly altered in the temperature range used in *Drosophila* follicle cells, or propose an alternative experimental approach (e.g., a partial loss-of-function allele of mud) that would more cleanly test the threshold prediction.

8. Assumption of uniform FG distribution in zebrafish endothelial cells (Line 385)

The authors acknowledge that the localization of cortical force generators in zebrafish ISV endothelial cells has not been determined (line 387). They then proceed to model the "simplest" case as a uniform distribution of FGs around the cortex. However, this choice of prior is not well-justified. The overwhelming evidence from other systems (*Drosophila* epithelia, neuroblasts, *C. elegans*, mammalian cells) is that FGs are asymmetrically distributed. Beginning from a uniform distribution therefore represents an assumption that is contrary to the general biology, not a neutral starting point. The authors should explicitly justify why uniform distribution is the appropriate null hypothesis here, rather than, for example, a distribution biased toward the proximal junction (which is where VE-Cadherin and ZO-1 are enriched, as the authors themselves show in Figure 6D). This choice has a material effect on the interpretation of Figure 6B-6C: the fact that uniform FG conditions fail to recapitulate spindle position may simply reflect that the uniform model is a poor biological prior rather than a meaningful mechanistic test.

Minor Comments

Line 297-299: The authors correctly note that the apparent trend in the pushing-only ensemble average is an artifact of averaging stochastic paths. This point could be made more visually obvious by showing individual trajectories (which the authors say are in Supplemental Figure 4A) directly in the main text figure, or at least referencing this panel more prominently here.

Line 302-304: The authors note that spindle alignment occurs faster in their model than in chick neuroepithelial data (Peyre et al., 2011) and attribute this to underestimation of cytoplasmic drag or overestimation of cortical forces. This discrepancy deserves more attention, are the timescales qualitatively or quantitatively different? A brief quantitative comparison would clarify whether this is a minor calibration issue or a more substantive model limitation.

Line 380: "<45" and "<30" should include degree symbols for consistency with the rest of the manuscript.

Line 382: "~3.5m" should read "~3.5 μm " (missing micron symbol).

Figure 4 legend: The legend states "Boxplots as in (C)" for panel E, but panel C is a simulation snapshot, not a boxplot. This appears to be a mislabeling and should reference panel D instead.

Figure 5A' (microscopy panel): The time stamp "231" appears twice in the image sequence (frames 6 and 7 as labelled), suggesting a labeling error in the figure. This should be checked and corrected.

The simulation of *C. elegans* spindle posterior displacement (Figure 3B) does not incorporate spindle elongation, which is known to be critical for accurate posterior positioning in this system. While the authors note this limitation, they should more explicitly discuss what the model can and cannot conclude about the *C. elegans* positioning mechanism given this omission.

The Limitations section (lines 433-450) is commendably thorough, but the omission of cytoplasmic pulling forces (line 448) is listed as an afterthought. Given that cytoplasmic pulling has been specifically shown to contribute to centering in *C. elegans* (Kimura and Kimura 2011; Kimura and Onami 2005), this limitation is particularly relevant for the *C. elegans* section and should be discussed there rather than only in the Limitations paragraph.

Referee #2 (Report for Author)

Yeltokov et al. have constructed a computational model of spindle orientation and positioning involving microtubule pushing against the cell cortex, and pulling with the help of cortical force generators such as the dynein molecular motors. They apply this model to

explain spindle orientation and positioning in a range of asymmetrically dividing cells, from classical model systems such as *Drosophila* S2 cells and the first division of a *C. elegans* embryo, to less well studied and more heterogeneous Zebrafish endothelial cells. In the latter model, the authors use a combination of image segmentation and computational simulations to compare the experimental time-lapse movies of spindle orientation establishment with the results of a computational model provided the cell shape as the input. The authors then vary parameters of their model to analyse the contributions of microtubule pushing, cortical pulling, and the spatial distribution of the cortical force generators to the observed similarity between experimental and simulated spindle orientations. The authors conclude that in this Zebrafish system, microtubule pushing forces mainly contribute to spindle orientation, while asymmetrical pulling forces mainly contribute to spindle positioning.

I found the paper written in an accessible manner for people without mathematics/physics background like myself, with clear figures. The strongest part of the reported data are the side-by-side comparisons between experiments and simulations for dynamically reshaping, irregular cells undergoing asymmetric division (reported in Fig 5-6) - this is certainly novel and interesting. I found other parts of the paper less convincing/interesting, and I have several suggestions to improve the strength of the main arguments.

1. For comparisons between experiments and simulations in Fig 5-6, the authors report only the difference between the experiment and the simulation for both the spindle angle and the spindle position. It is unclear what is an acceptable $\Delta(\text{obs-model})$ value? Perhaps better would be to directly report the distributions of experimental values, and separately for simulation values for each condition tested, such as in Fig 6FG. This will be important for the readers to get a better understanding of inherent variability of spindle orientations and positions, and of the model's performance in reproducing these experimental values. The authors could then compare the simulations with the experimental observations directly - as opposed to the currently reported comparisons of $\Delta(\text{obs-model})$ for different model configurations. Additionally, are the results of the simulation robust over several runs with the same shape of the cell? Reporting this directly, in my opinion, would be more informative than the current presentation where only the $\Delta(\text{obs-model})$ values are reported, not the absolute values per run/per cell.

2. The authors make an attempt to produce a unified model with a single parameter set that they then apply to a range of experimental model systems. I wonder if they checked how sensitive are their results to microtubule growth/shortening speeds and frequencies of rescue/catastrophe? Currently, the four parameters describing microtubule dynamic

instability are taken from two experimental reports - one on BS-C-1 cells from the African green monkey (Burakov JCB 2003), the other on *Drosophila* S2 cells (Rogers JCB 2002), and one theoretical study on *C. elegans* (Kimura & Onami Dev Cell 2005, which in turn derives these parameters from other experimental studies on a range of models from newt lung cells to *Xenopus* extracts). However, these dynamic parameters can be different in different experimental systems. In *C. elegans* the microtubule dynamics can be quite variable and the values reported, for example in Lacroix Dev Cell 2018, differ by several fold from the values used in the model. Are these parameters known for Zebrafish endothelial cells?

3. I found the title slightly misleading. At the end of their Results section the authors conclude that "an asymmetric pulling force ... required to correctly position the spindle", based on results reported in Fig 6. This is a little hard for me to reconcile with "beyond pulling" in the title?

4. There are quite a few computational models reported in the literature that consider the contributions of both microtubule pulling and pushing forces in a variety of systems - some the authors have cited (Letort MBoC 2016, Kozlowski Cell 2007), some not (such as Pavin New J Phys 2012 and related papers). Perhaps the authors may wish to expand their quite compact Discussion to place their new model and results in the context of these previously published models - this will help a broader audience to appreciate what is novel in the current work. Since the authors consider their spindle to be simply a passive object being positioned, from the modelling point of view it is quite similar to a centrosome/MTOC and so the authors could use models that consider aster positioning for comparison.

Minor comments:

5. In experiments reported in Supplementary Fig 2, are there any force generators left at the cortex, or is it known how strongly are they depleted compared to normal levels? What would be the outcome of the simulations reported in Fig2D/D' in a condition with zero FG?

6. Results reported in Fig4 suggest that the "pulling only" model works reasonably well in allowing the spindle to eventually reach the normal orientation. The "pushing only" model, on the other hand, performs poorly. To me this result seems to contradict the data in Fig 2D/D' where the model with a decreasing number of FGs works increasingly bad, while the remaining pushing forces seem to be sufficient? Is there something I miss in these interpretations?

Referee #3 (Report for Author)

Yeltokov et al. developed a 2D agent-based computational model integrating cortical dynein-mediated pulling forces and MT polymerization-based pushing forces to study mitotic spindle orientation and positioning (Fig. 1). The central claim is that this constitutes a "generalizable" framework applicable across diverse cell types, in contrast to prior cell-type-specific models. The model is validated against established experimental observations in *Drosophila* follicular epithelial cells and neuroblasts (Fig. 2) and in the *C. elegans* embryo (Fig.3), and is then applied to zebrafish angiogenic endothelial tip cells (Figs. 5, 6). The principal scientific conclusions are that MT pushing forces act as geometric sensors of cell shape, prevent spindle stalling by buffering poles away from the cortex, and promote spindle dynamicity-roles proposed to be complementary to, rather than redundant with, cortical pulling.

The ambition to apply a single unified modeling framework across multiple organisms and cell types is commendable and represents a valuable direction for the field. However, for such a framework to support the conclusions drawn here, the model must be adequate for the specific claims being made. As detailed below, I have serious concerns that the current model lacks key components necessary to substantiate the claim of generalizability and, as a consequence, the scientific conclusions regarding the role of MT pushing forces cannot be adequately evaluated.

Major Concerns

#1: The 2D formulation undermines the claim of generalizability

The model operates in a 2D midplane cross-section, in which the cortex is represented as a 1D boundary. In a real 3D cell, the cortex is a 2D surface, and the number, angular distribution, and force balance of MT-cortex interactions are qualitatively different. This distinction is especially consequential here, where cortical interactions are the central subject of inquiry. The authors' rationale for using 2D-that experimental data are often available only in 2D-does not hold, since 3D simulations can be compared with 2D data via projection.

Importantly, 3D models of comparable complexity to the present work already exist in the cortical pulling literature. For example, Kimura & Onami (*J Cell Biol* 179, 1347-1354, 2007) and Farhadifar et al. (*eLife* 9, e55877, 2020)-neither of which is cited in the present manuscript-demonstrate that 3D formulations are tractable at a similar level of modeling complexity. The current 2D framework therefore does not represent a technical advance in this respect.

I consider 3D validation of the principal conclusions, particularly those concerning cell-shape-dependent pushing forces and the zebrafish endothelial cell application, to be necessary before the generalizable nature of this model can be accepted.

#2: Exclusion of cytoplasmic pulling forces undermines the primary scientific conclusions
The authors exclude cytoplasmic pulling forces-length-dependent forces exerted by cytoplasmic dynein throughout the cell volume-on the grounds that cortical interactions dominate spindle behavior. This exclusion is not adequately justified. It is well established that cytoplasmic pulling forces can encode cell shape information and direct spindle orientation (e.g., Minc et al. Cell 144, 414-426, 2011; Kimura & Onami 2007), overlapping substantially with the "geometric sensor" function attributed to MT pushing in this manuscript. A model that excludes cytoplasmic pulling cannot distinguish between the contributions of pushing and cytoplasmic pulling, and therefore cannot establish that the effects attributed to pushing are not in fact mediated by cytoplasmic pulling. Demonstrating that the proposed role of MT pushing cannot be fulfilled by cytoplasmic pulling forces is essential to the central argument of this paper, and this comparison is entirely absent. A model claiming generalizability should incorporate both cortical and cytoplasmic force-generating mechanisms.

Minor Concerns

#3: Advantages over existing models are not articulated

The authors position their model as a generalizable alternative to prior cell-type-specific models, but do not explain what the present framework offers beyond existing approaches. Many prior models are themselves extensible in principle, and it is not clear what technical or conceptual innovations enable the claimed generalizability here. The authors should explicitly compare their framework with the most relevant prior models and articulate where and why the present approach offers advantages. This is particularly important given that the current model is, in some respects-dimensionality, force mechanisms included-less complete than existing formulations.

#4: Sensitivity of conclusions to MT length distribution

Pushing forces in the model are inversely proportional to the square of MT length, making the relative contributions of pushing and pulling-and thus the principal conclusions-potentially sensitive to the assumed MT length distribution. While MT number and FG number are varied systematically, MT length distribution is not. The authors should identify which conclusions are robust across a range of length distributions and which are not. Given the strong length dependence of the pushing force, this analysis is necessary to assess the reliability of the quantitative predictions.

#5: Accuracy of descriptions of prior work

In the *C. elegans* sections, I identified at least one statement that appears inaccurate. At L.282-284, the authors state that spindle orientation "generally fails" after disruption of the canonical pulling machinery. This overstates the phenotype: it is well documented that spindle orientation is not entirely abrogated under these conditions in *C. elegans*, and the relationship is more nuanced than implied. I raise this as an example rather than an isolated concern—given the breadth of systems covered in this manuscript, I would ask the authors to carefully review all descriptions of prior experimental and computational work for accuracy, and to ensure that the novelty of the present contributions is clearly delineated from what has already been established.

Revision Plan for EMBOJ-2026-124185 (Yeltokov et al.)

We are grateful to the editor and the reviewers for the thoughtful and detailed evaluation of our manuscript. We found the critiques highly constructive and believe they will substantially improve both the rigor and clarity of the work. We were encouraged that the reviewers recognized the value of the cross-system approach and especially the application to dynamically reshaping endothelial cells. Following extensive discussion between the authors, we believe the major concerns raised can be effectively addressed through reframing of the manuscript, expanded discussion of prior literature and model limitations, additional sensitivity analyses, and targeted new simulations. Importantly, while the reviewers raised questions about model scope and interpretation, they did not challenge the broader conclusion that MT pushing can contribute alongside cortical pulling forces during spindle orientation and positioning. We are therefore optimistic that the revised manuscript will provide a substantially strengthened and more clearly contextualized framework for understanding complementary force contributions during spindle dynamics.

Below we outline our point-by-point revision plan.

Referee #1 (Report for Author)**Summary**

This manuscript presents a generalizable 2D computational model incorporating both cortical microtubule (MT) pulling and polymerization-based pushing forces to investigate mitotic spindle orientation and positioning across diverse cell types. The model is validated against multiple experimental systems, such as *Drosophila* follicular epithelial cells, neuroblasts, and the *C. elegans* embryo, before being applied to the more challenging case of irregularly shaped zebrafish intersegmental vessel (ISV) endothelial cells. The authors conclude that pulling forces are the primary determinants of directed spindle orientation, while pushing forces play essential supporting roles by preventing spindle stalling, promoting dynamicity, and, in the zebrafish context, encoding cell geometry to drive spindle orientation independently of pulling. The paper is clearly written, the model is thoughtfully constructed, and the biological scope is impressive. However, several points require clarification or additional justification.

Major Comments

1. Missing citation for laser ablation evidence (Line 41): The claim at line 41 that laser ablation of astral MTs triggers spindle recoil is supported by citations to Bosveld et al. (2016) and Wu et al. (2024). However, the foundational laser ablation experiments demonstrating pulling forces on centrosomes by Stephan Grill and colleagues (Grill et al., 2001, *Nature*, and related work) should be cited here, as this is the primary evidence for cortical pulling forces in the field. The current citation list gives the impression this is a recent discovery rather than a well-established result.

We thank the reviewer for pointing out this omission. We agree that Grill et al. (2001) is the foundational work demonstrating cortical pulling forces in this context. We will revise the text to include this citation.

2. Figure 1D and 1E: Dynamic instability of MTs not represented (Figure 1D, 1E): In Figure 1D, the pushing MT is depicted as a static filament, with no indication that it is in a growing (polymerizing) state. This is biologically important because pushing forces arise specifically from growing MT plus-ends encountering the cortex. A shrinking MT would not generate a pushing

force. The authors explicitly state in the text (line 140-141) that pushing occurs when a "growing MT reaches the cortex," yet the figure does not convey this. The same issue applies to Figure 1E, the schematic should distinguish growing (pushing) from shrinking MTs more clearly in the context of force generation, consistent with the color-coding introduced later in Figure 2B. The authors should either add a polymerization indicator (e.g., growing tip markers) or an explicit label to clarify that this depicts a growing MT.

We agree with the reviewer's assessment. We will revise Figures 1D and 1E to clearly reflect the dynamic instability of the MTs. Specifically, we will add growing tip markers to represent the polymerizing state of the pushing MTs and update the color-coding to ensure consistency with Figure 2B.

3. Model parameters: MT and force generator numbers require clearer justification. The authors use astral MT numbers scaled from *C. elegans* estimates (500-1000 per aster; Kozlowski et al., 2007) and a range of 40-400 force generators (FGs) citing Grill et al. (2003). The manuscript justifies its FG range of 40-400 citing Grill et al. (2003) for the estimate that ~50 FGs are required for spindle positioning. Pécrciaux et al. (2006) uses $N = 28$ motors per side of the AP axis (~56 total) in their model of anaphase oscillations in *C. elegans*, which is broadly consistent with the lower end of the range. However, both estimates come from anaphase dynamics in *C. elegans* specifically. The authors should clarify whether these numbers are appropriate starting points for the smaller *Drosophila* cells, where the range of 40-400 spans an order of magnitude without clear biological justification for the upper bound.

We thank the reviewer for highlighting the need for clearer justification regarding our parameter choices.

First, we note a typographical error in the manuscript: the force generator (FG) range used in all simulations and figures is actually **20-200**, not 40-400. We apologize for the confusion and will correct this in the revised manuscript.

Since direct measurements for these parameters are currently unavailable in *Drosophila* follicle cells or zebrafish, we scaled our values from established *C. elegans* benchmarks as follows:

- *C. elegans* studies estimate that ~50 FGs are required for spindle positioning (Grill et al., 2003; Pécrciaux et al., 2006). Since *Drosophila* follicle cells are substantially smaller, we chose a conservative lower bound of 20 FGs. The upper bound of 200 FGs represents a fourfold excess over the *C. elegans* baseline; this was chosen not to model a specific biological condition, but to systematically explore how high FG density influences spindle orientation dynamics (as reported in Figure 2D).
- With regard to astral MT numbers, please refer to our response to comment #5.

We will update the Methods section of the manuscript to explicitly include these biological rationales.

4. The spatial distribution of motors/FGs on the cortex is a critical parameter that shapes the outcome but is not systematically discussed. How sensitive are the results to the assumed spatial arrangement of FGs, particularly in the neuroblast and follicle cell conditions? The FG distribution is derived from fluorescence intensity profiles (Mud signal), but the mapping from fluorescence intensity to motor number is not validated. A sensitivity analysis varying FG distribution shape (e.g., width and peak position of the gradient) would substantially strengthen the modeling conclusions.

We agree that characterizing the systems' sensitivity to the spatial distribution of FGs is crucial, particularly given that mapping fluorescence intensity (Mud signal) to absolute motor numbers carries inherent uncertainties. In the revised manuscript, we will include a systematic sensitivity analysis for both follicle cells and neuroblasts. Specifically, we will vary the key parameters of the FG gradient – including distribution width, peak position, and steepness – and evaluate their specific impacts on first passage time and alignment success rate.

5. For *Drosophila* cells, the MT numbers used in simulations (50-400 in Figure 2D) are substantially below the *C. elegans* scaling estimate. The justification for this range in *Drosophila* specifically should be stated.

We thank the reviewer for this constructive comment. Since *Drosophila* cells are significantly smaller than *C. elegans* embryos, we scaled down the astral MT numbers proportionally to cell size. This is consistent with estimates of ~20 cortex-contacting astral MTs in human mitotic cells of a comparable size (Anjur-Dietrich et al., 2024). We chose the 50-400 range to establish a biologically plausible baseline at the lower end while systematically exploring the broader effects of astral MT number on spindle orientation dynamics (Figure 2D). We will update the Methods section of our manuscript to explicitly include this biological rationale.

[https://www.cell.com/developmental-cell/fulltext/S1534-5807\(24\)00340-X](https://www.cell.com/developmental-cell/fulltext/S1534-5807(24)00340-X)

6. Tension with Pécrcéaux et al. (2016) on the role of pushing vs. pulling during *C. elegans* metaphase (Figure 3B): Pécrcéaux et al. (2016) directly addresses spindle centering during metaphase in the *C. elegans* one-cell embryo. That paper independently estimates that ~1000 active force-generating elements underlie metaphase centering, which is consistent with the MT numbers used by Yeltokov et al. (500-1000 per aster from Kozlowski et al. 2007). However, Pécrcéaux et al. (2016) reach a mechanistically important conclusion, they propose that the high precision of metaphase centering is accounted for quantitatively by MT pushing alone, and cortical pulling forces are argued to become dominant only during anaphase. This creates a direct tension with the Yeltokov model, which simultaneously incorporates both cortical pulling and pushing forces during the phase modelled in Figure 3B. This tension is not acknowledged or discussed anywhere in the manuscript. The authors cite Pécrcéaux et al. (2016) only for simulation duration parameters (line 151), while overlooking its mechanistic conclusions that are directly relevant to their modeling choices. They should address this contradiction. Without this discussion, the paper's claim to provide a "unified framework" for *C. elegans* spindle dynamics is incomplete.

We agree this needs to be addressed explicitly. In revision, we will expand our discussion of metaphase centering models and clarify how our framework relates to prior pushing-based centering models versus pulling-dominated regimes. We will include simulations for PNC centering in pushing-only condition to reconcile our work with Pécrcéaux et al. (2016).

7. Experimental validation of threshold behavior (Lines 216-225): The authors attempt to validate the model's predicted threshold behavior by depleting Mud using Traffic jam-GAL4 / UAS-mud-shRNA at varying temperatures (25°C, 27°C, 29°C). While the results are consistent with the model prediction, there is a significant confound, temperature affects not only GAL4 activity (and thus Mud levels / FG number) but also MT dynamics, including polymerization rate, catastrophe frequency, and rescue rate. This means the experiment is not cleanly changing a single parameter. The authors acknowledge none of this. At minimum, they should discuss this limitation explicitly and note that the temperature-dependent effects on MT

dynamics could independently alter spindle behavior. Ideally, they would either cite evidence that MT dynamics are not significantly altered in the temperature range used in *Drosophila* follicle cells, or propose an alternative experimental approach (e.g., a partial loss-of-function allele of *mud*) that would more cleanly test the threshold prediction.

This is an interesting point! We will explicitly acknowledge temperature effects on MT dynamics and more carefully frame the experiment as consistent with, but not a definitive test of, the predicted threshold behavior. The reviewer will appreciate that a hypomorphic allele is not ideal in this instance, as any phenotype (or lack thereof) can be interpreted as either reflecting the underlying biology or simply an insufficient reduction in Mud activity, making it impossible to draw any conclusions about threshold behavior. However, we are very willing to try a genetic reduction (loss of one copy).

8. Assumption of uniform FG distribution in zebrafish endothelial cells (Line 385): The authors acknowledge that the localization of cortical force generators in zebrafish ISV endothelial cells has not been determined (line 387). They then proceed to model the "simplest" case as a uniform distribution of FGs around the cortex. However, this choice of prior is not well-justified. The overwhelming evidence from other systems (*Drosophila* epithelia, neuroblasts, *C. elegans*, mammalian cells) is that FGs are asymmetrically distributed. Beginning from a uniform distribution therefore represents an assumption that is contrary to the general biology, not a neutral starting point. The authors should explicitly justify why uniform distribution is the appropriate null hypothesis here, rather than, for example, a distribution biased toward the proximal junction (which is where VE-Cadherin and ZO-1 are enriched, as the authors themselves show in Figure 6D). This choice has a material effect on the interpretation of Figure 6B-6C: the fact that uniform FG conditions fail to recapitulate spindle position may simply reflect that the uniform model is a poor biological prior rather than a meaningful mechanistic test.

We agree that this assumption requires stronger justification. In revision, we will:

- clarify that this represents a minimal/null model,
- test alternative FG distributions (including junctional bias),
- and more clearly frame the biological interpretation.

Minor Comments

Line 297-299: The authors correctly note that the apparent trend in the pushing-only ensemble average is an artifact of averaging stochastic paths. This point could be made more visually obvious by showing individual trajectories (which the authors say are in Supplemental Figure 4A) directly in the main text figure, or at least referencing this panel more prominently here.

We thank the reviewer for this suggestion. In the revised manuscript we will make this point more visually apparent.

Line 302-304: The authors note that spindle alignment occurs faster in their model than in chick neuroepithelial data (Peyre et al., 2011) and attribute this to underestimation of cytoplasmic drag or overestimation of cortical forces. This discrepancy deserves more attention, are the timescales qualitatively or quantitatively different? A brief quantitative comparison would clarify whether this is a minor calibration issue or a more substantive model limitation.

We thank the reviewer for this point. In the revised manuscript we will include a comparison of modeled and reported alignment timescales from Peyre et al. (2011) to clarify whether the discrepancy is related to parameter calibration or model physics.

Line 380: "<45" and "<30" should include degree symbols for consistency with the rest of the manuscript.

Good catch, thanks! We have added those degree symbols.

Line 382: "~3.5m" should read "~3.5 μm " (missing micron symbol).

Thanks. We have replaced "~3.5m" with "~3.5 μm ".

Figure 4 legend: The legend states "Boxplots as in (C)" for panel E, but panel C is a simulation snapshot, not a boxplot. This appears to be a mislabeling and should reference panel D instead.

The reference "Boxplots as in (C)" is indeed incorrect. We have corrected this to reference panel D in the revised legend.

Figure 5A' (microscopy panel): The time stamp "231" appears twice in the image sequence (frames 6 and 7 as labelled), suggesting a labeling error in the figure. This should be checked and corrected.

The reviewer is right! We've corrected the error.

The simulation of *C. elegans* spindle posterior displacement (Figure 3B) does not incorporate spindle elongation, which is known to be critical for accurate posterior positioning in this system. While the authors note this limitation, they should more explicitly discuss what the model can and cannot conclude about the *C. elegans* positioning mechanism given this omission.

We thank reviewer for this comment. We will discuss in detail how the limitation of our model affects interpretation of spindle positioning results.

The Limitations section (lines 433-450) is commendably thorough, but the omission of cytoplasmic pulling forces (line 448) is listed as an afterthought. Given that cytoplasmic pulling has been specifically shown to contribute to centering in *C. elegans* (Kimura and Kimura 2011; Kimura and Onami 2005), this limitation is particularly relevant for the *C. elegans* section and should be discussed there rather than only in the Limitations paragraph.

The third reviewer raised a similar issue (their Comment #2). In light of that concern, we intend to incorporate cytoplasmic pulling forces into our model and will therefore have more to say in the revised manuscript.

Referee #2 (Report for Author)

Yeltokov *et al.* have constructed a computational model of spindle orientation and positioning involving microtubule pushing against the cell cortex and pulling with the help of cortical force generators such as the dynein molecular motors. They apply this model to explain spindle orientation and positioning in a range of asymmetrically dividing cells, from classical model systems such as *Drosophila* S2 cells and the first division of a *C. elegans* embryo, to less well studied and more heterogeneous Zebrafish endothelial cells. In the latter model, the authors use a combination of image segmentation and computational simulations to compare the experimental time-lapse movies of spindle orientation establishment with the results of a computational model

provided the cell shape as the input. The authors then vary parameters of their model to analyse the contributions of microtubule pushing, cortical pulling, and the spatial distribution of the cortical force generators to the observed similarity between experimental and simulated spindle orientations. The authors conclude that in this Zebrafish system, microtubule pushing forces mainly contribute to spindle orientation, while asymmetrical pulling forces mainly contribute to spindle positioning.

I found the paper written in an accessible manner for people without mathematics/physics background like myself, with clear figures. The strongest part of the reported data are the side-by-side comparisons between experiments and simulations for dynamically reshaping, irregular cells undergoing asymmetric division (reported in Fig 5-6) - this is certainly novel and interesting. I found other parts of the paper less convincing/interesting, and I have several suggestions to improve the strength of the main arguments.

1. For comparisons between experiments and simulations in Fig 5-6, the authors report only the difference between the experiment and the simulation for both the spindle angle and the spindle position. It is unclear what is an acceptable $\Delta(\text{obs-model})$ value? Perhaps better would be to directly report the distributions of experimental values, and separately for simulation values for each condition tested, such as in Fig 6FG. This will be important for the readers to get a better understanding of inherent variability of spindle orientations and positions, and of the model's performance in reproducing these experimental values. The authors could then compare the simulations with the experimental observations directly - as opposed to the currently reported comparisons of $\Delta(\text{obs-model})$ for different model configurations. Additionally, are the results of the simulation robust over several runs with the same shape of the cell? Reporting this directly, in my opinion, would be more informative than the current presentation where only the $\Delta(\text{obs-model})$ values are reported, not the absolute values per run/per cell.

We agree that clearer presentation of simulation robustness and direct comparison with experimental values would strengthen the figures. We note that plotting absolute values across cells is not straightforward: the 12 cells differ in size and shape, and the degree of spindle displacement varies between cells without a clear trend, meaning raw position values are not directly comparable across cells. We will explore appropriate normalization strategies to enable direct cross-cell comparisons and will revise the figures accordingly. We draw the reviewer's attention to Supplemental Figure 7, where simulation results for every individual cell under each condition are already presented, with each bar plot representing 40 simulation runs. These data directly address the question of within-cell simulation robustness.

2. The authors make an attempt to produce a unified model with a single parameter set that they then apply to a range of experimental model systems. I wonder if they checked how sensitive are their results to microtubule growth/shortening speeds and frequencies of rescue/catastrophe? Currently, the four parameters describing microtubule dynamic instability are taken from two experimental reports - one on BS-C-1 cells from the African green monkey (Burakov JCB 2003), the other on *Drosophila* S2 cells (Rogers JCB 2002), and one theoretical study on *C. elegans* (Kimura & Onami Dev Cell 2005, which in turn derives these parameters from other experimental studies on a range of models from newt lung cells to *Xenopus* extracts). However, these dynamic parameters can be different in different experimental systems. In *C. elegans* the microtubule dynamics can be quite variable and the values reported, for example in Lacroix Dev Cell 2018, differ by several fold from the values used in the model. Are these parameters known for Zebrafish endothelial cells?

We are currently performing additional analyses with varying growth/shrinkage rates and catastrophe/rescue frequencies to assess robustness. These results will be incorporated into the revision.

3. I found the title slightly misleading. At the end of their Results section the authors conclude that "an asymmetric pulling force ... required to correctly position the spindle", based on results reported in Fig 6. This is a little hard for me to reconcile with "beyond pulling" in the title?

We appreciate the reviewer's point and are happy to change the title. We like "Microtubule pushing and cortical pulling cooperate to promote robust spindle orientation" as an alternative.

4. There are quite a few computational models reported in the literature that consider the contributions of both microtubule pulling and pushing forces in a variety of systems - some the authors have cited (Letort MBoC 2016, Kozlowski Cell 2007), some not (such as Pavin New J Phys 2012 and related papers). Perhaps the authors may wish to expand their quite compact Discussion to place their new model and results in the context of these previously published models - this will help a broader audience to appreciate what is novel in the current work. Since the authors consider their spindle to be simply a passive object being positioned, from the modelling point of view it is quite similar to a centrosome/MTOC and so the authors could use models that consider aster positioning for comparison.

We will substantially expand the Discussion to more clearly situate this work within the broader modeling literature, including additional references suggested by the reviewer.

Minor comments:

5. In experiments reported in Supplementary Fig 2, are there any force generators left at the cortex, or is it known how strongly are they depleted compared to normal levels? What would be the outcome of the simulations reported in Fig2D/D' in a condition with zero FG?

Unfortunately, we do not know how much Mud is left at the cortex in these experiments. We do have an anti-Mud antibody but it is not very sensitive and we are therefore not confident making comparisons based on signal intensity. A weaker signal could be a genuine result or reflect normal variability when using this reagent.

A condition with zero FGs corresponds to the pushing-only regime. In follicle cells, pushing forces are isotropic — they promote spindle movement but provide no directional bias toward the correct orientation. As a result, spindle angle trajectories in this condition are effectively randomized, and first passage time cannot be meaningfully calculated or included in the Figure 2D/D' heatmap.

6. Results reported in Fig4 suggest that the "pulling only" model works reasonably well in allowing the spindle to eventually reach the normal orientation. The "pushing only" model, on the other hand, performs poorly. To me this result seems to contradict the data in Fig 2D/D' where the model with a decreasing number of FGs works increasingly bad, while the remaining pushing forces seem to be sufficient? Is there something I miss in these interpretations?

We regret this confusion, which appears to stem from lack of clarity about the conditions used in each figure. Figure 2 simulations are run exclusively in the combined pulling and pushing condition — reducing FG number weakens pulling forces but never eliminates them. Even at the lowest FG numbers shown, residual pulling forces remain present and continue to drive orientation, but less efficiently. This is not equivalent to the pushing-only condition in Figure 4,

where cortical pulling is entirely absent. The two figures are fully consistent: pulling is the primary driver of orientation, and its partial reduction slows but does not abolish alignment, while its complete removal causes orientation to fail. In the revised manuscript we will add an explicit statement at the start of the validation section clarifying that all simulations there use the combined condition, and will note that the dissection of individual force contributions is addressed subsequently in Figure 4.

Referee #3 (Report for Author)

Yeltokov et al. developed a 2D agent-based computational model integrating cortical dynein-mediated pulling forces and MT polymerization-based pushing forces to study mitotic spindle orientation and positioning (Fig. 1). The central claim is that this constitutes a "generalizable" framework applicable across diverse cell types, in contrast to prior cell-type-specific models. The model is validated against established experimental observations in *Drosophila* follicular epithelial cells and neuroblasts (Fig. 2) and in the *C. elegans* embryo (Fig. 3), and is then applied to zebrafish angiogenic endothelial tip cells (Figs. 5, 6). The principal scientific conclusions are that MT pushing forces act as geometric sensors of cell shape, prevent spindle stalling by buffering poles away from the cortex, and promote spindle dynamics-roles proposed to be complementary to, rather than redundant with, cortical pulling.

The ambition to apply a single unified modeling framework across multiple organisms and cell types is commendable and represents a valuable direction for the field. However, for such a framework to support the conclusions drawn here, the model must be adequate for the specific claims being made. As detailed below, I have serious concerns that the current model lacks key components necessary to substantiate the claim of generalizability and, as a consequence, the scientific conclusions regarding the role of MT pushing forces cannot be adequately evaluated.

Major Concerns

#1: The 2D formulation undermines the claim of generalizability: The model operates in a 2D midplane cross-section, in which the cortex is represented as a 1D boundary. In a real 3D cell, the cortex is a 2D surface, and the number, angular distribution, and force balance of MT-cortex interactions are qualitatively different. This distinction is especially consequential here, where cortical interactions are the central subject of inquiry. The authors' rationale for using 2D—that experimental data are often available only in 2D—does not hold, since 3D simulations can be compared with 2D data via projection.

Importantly, 3D models of comparable complexity to the present work already exist in the cortical pulling literature. For example, Kimura & Onami (J Cell Biol 179, 1347-1354, 2007) and Farhadifar et al. (eLife 9, e55877, 2020)—neither of which is cited in the present manuscript—demonstrate that 3D formulations are tractable at a similar level of modeling complexity. The current 2D framework therefore does not represent a technical advance in this respect.

I consider 3D validation of the principal conclusions, particularly those concerning cell-shape-dependent pushing forces and the zebrafish endothelial cell application, to be necessary before the generalizable nature of this model can be accepted.

We thank the reviewer for this constructive critique regarding the dimensionality of our computational model. We agree that moving from a 2D midplane approximation to a full 3D surface alters the absolute number, angular distribution, and precise force balance of MT-cortex

interactions. We will revise the Abstract, Introduction, and Discussion to moderate our claims of generalizability. Specifically, we will clarify that the current model is a formalized 2D midplane framework designed to explore specific geometric principles, rather than a definitive 3D representation of all cortical interactions.

We also appreciate the reviewer highlighting the omission of Kimura & Onami (2007) and Farhadifar et al. (2020). We will cite both papers in the revised Discussion to provide a more comprehensive overview of the cortical interaction landscape. However, we would like to clarify the distinct utility and novelty of our 2D framework relative to these prior studies.

Although Kimura & Onami (2007) and Farhadifar et al. (2020) elegantly demonstrate the tractability of 3D formulations, these models primarily focus on cortical pulling mechanisms and astral MT length dynamics during centrosome/spindle positioning. In contrast, our work specifically examines cell-shape-dependent pushing forces and geometric boundary constraints. The mechanical constraints governing MT buckling and pushing within a confined midplane therefore represent a distinct physical regime.

While 3D models can be projected for comparison with 2D data, fully resolved 3D simulations of complex, dynamic, and highly asymmetric geometries – such as the zebrafish endothelial cells studied here – introduce substantial additional degrees of freedom and unconstrained parameters related to cortical heterogeneity. By contrast, our 2D framework enables tighter, high-throughput exploration of parameter space while isolating the role of boundary curvature within a single plane, without the additional complexity introduced by 3D cortical anisotropy.

We believe that clearly delineating these limitations, acknowledging the insights provided by prior 3D models, and moderating our claims of generalizability will better contextualize both the novelty and the utility of our 2D framework.

#2: Exclusion of cytoplasmic pulling forces undermines the primary scientific conclusions

The authors exclude cytoplasmic pulling forces-length-dependent forces exerted by cytoplasmic dynein throughout the cell volume-on the grounds that cortical interactions dominate spindle behavior. This exclusion is not adequately justified. It is well established that cytoplasmic pulling forces can encode cell shape information and direct spindle orientation (e.g., Minc et al. Cell 144, 414-426, 2011; Kimura & Onami 2007), overlapping substantially with the "geometric sensor" function attributed to MT pushing in this manuscript. A model that excludes cytoplasmic pulling cannot distinguish between the contributions of pushing and cytoplasmic pulling, and therefore cannot establish that the effects attributed to pushing are not in fact mediated by cytoplasmic pulling. Demonstrating that the proposed role of MT pushing cannot be fulfilled by cytoplasmic pulling forces is essential to the central argument of this paper, and this comparison is entirely absent. A model claiming generalizability should incorporate both cortical and cytoplasmic force-generating mechanisms.

We thank the reviewer for this insightful critique. We agree that length-dependent cytoplasmic pulling is a well-established mechanism for cell shape sensing (Kimura & Onami, 2007; Minc et al., 2011) that overlaps conceptually with our proposed MT pushing model. To address this and robustly decouple the effects of pushing versus pulling, we will update our model to include a volumetric pulling force proportional to MT length. This will allow us to directly compare the two mechanisms within our specific cellular geometries. We will incorporate these new comparative simulations into the revised manuscript to explicitly test whether cytoplasmic pulling can fulfill the role we have attributed to MT pushing, thereby ensuring our model's broader generalizability.

Minor Concerns

#3: Advantages over existing models are not articulated

The authors position their model as a generalizable alternative to prior cell-type-specific models, but do not explain what the present framework offers beyond existing approaches. Many prior models are themselves extensible in principle, and it is not clear what technical or conceptual innovations enable the claimed generalizability here. The authors should explicitly compare their framework with the most relevant prior models and articulate where and why the present approach offers advantages. This is particularly important given that the current model is, in some respects-dimensionality, force mechanisms included-less complete than existing formulations.

We will substantially expand the Discussion to clarify how the present framework differs from and relates to previous models, including both 2D and 3D approaches. To summarize, the present framework offers the following advances over existing models: it systematically compares pushing-alone, pulling-alone, and combined force regimes within a single framework; and it incorporates temporally evolving cell shapes from live imaging data, enabling simulation of non-rounding cells - a regime not previously addressed computationally.

#4: Sensitivity of conclusions to MT length distribution

Pushing forces in the model are inversely proportional to the square of MT length, making the relative contributions of pushing and pulling-and thus the principal conclusions-potentially sensitive to the assumed MT length distribution. While MT number and FG number are varied systematically, MT length distribution is not. The authors should identify which conclusions are robust across a range of length distributions and which are not. Given the strong length dependence of the pushing force, this analysis is necessary to assess the reliability of the quantitative predictions.

We thank the reviewer for this constructive critique. We agree that given the inverse-square dependence of pushing forces on MT length, the assumed MT length distribution is a critical factor that could influence the balance between pushing and pulling mechanisms. To test the reliability of our quantitative predictions, we will therefore perform an extensive sensitivity analysis by systematically varying the MT length distributions. Specifically, we will test a range of gamma distributions (chosen for their physiological plausibility as right-skewed, zero-bounded length distributions) by systematically varying the shape and scale parameters. We will add a detailed discussion of these additional simulations, along with insights gained regarding the robustness of our conclusions, to the revised manuscript.

#5: Accuracy of descriptions of prior work

In the *C. elegans* sections, I identified at least one statement that appears inaccurate. At L.282-284, the authors state that spindle orientation "generally fails" after disruption of the canonical pulling machinery. This overstates the phenotype: it is well documented that spindle orientation is not entirely abrogated under these conditions in *C. elegans*, and the relationship is more nuanced than implied. I raise this as an example rather than an isolated concern-given the breadth of systems covered in this manuscript, I would ask the authors to carefully review all descriptions of prior experimental and computational work for accuracy, and to ensure that the novelty of the present contributions is clearly delineated from what has already been established.

To address the reviewer's concern, we will carefully review all descriptions of prior experimental and computational work to ensure accuracy, while also refining the text to more clearly delineate the novelty and utility of our present contributions.

Dear Dan,

Thank you for submitting your manuscript for consideration by The EMBO Journal. We have now received reports from three reviewers, which I have included below.

As reflected in their reports, reviewers #1 and #2 are appreciative of the findings, while also indicating a number of concerns about the presented model that would need to be addressed before they can support publication of the study. On the other hand, reviewer #3 is critical on the contribution of the proposed model of spindle orientation regulation due to its 2D nature and the omitted contribution of pulling forces.

Based on the interest expressed in the reports of reviewer #1 and #2 and your willingness to engage in an extensive revision during the pre-decision consultation, I invite you to revise your manuscript as outlined in your revision plan.

We generally allow three months as standard revision time, which can be extended to six months in the case of major revisions. Should you foresee a problem in meeting this deadline, please let us know in advance to discuss an extension.

As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, please contact me as soon as possible upon publication of any related work to discuss the appropriate course of action.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File and will therefore be available online to the community. For more details on our Transparent Editorial Process, please review our Editorial Policies: <https://link.springer.com/partners/embo-press/editorial-policies>. Please also see the attached instructions for further guidelines on preparation of the revised manuscript.

Please feel free to contact me if you have any further questions regarding the revision. Thank you for the opportunity to consider your work for publication. I look forward to receiving the revised manuscript.

With best wishes,

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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 (20th Aug 2026). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

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Response Letter for EMBOJ-2026-124185 (Yeltokov et al.)

We are grateful to the editor and the reviewers for the thoughtful and detailed evaluation of our manuscript. We have taken the reviewers' concerns into account and believe that the manuscript is substantially improved. We are optimistic that this revised version provides a stronger and more clearly contextualized framework for understanding complementary force contributions during spindle dynamics. Our point-by-point responses are below:

Referee #1 (Report for Author)

Summary

This manuscript presents a generalizable 2D computational model incorporating both cortical microtubule (MT) pulling and polymerization-based pushing forces to investigate mitotic spindle orientation and positioning across diverse cell types. The model is validated against multiple experimental systems, such as *Drosophila* follicular epithelial cells, neuroblasts, and the *C. elegans* embryo, before being applied to the more challenging case of irregularly shaped zebrafish intersegmental vessel (ISV) endothelial cells. The authors conclude that pulling forces are the primary determinants of directed spindle orientation, while pushing forces play essential supporting roles by preventing spindle stalling, promoting dynamicity, and, in the zebrafish context, encoding cell geometry to drive spindle orientation independently of pulling. The paper is clearly written, the model is thoughtfully constructed, and the biological scope is impressive. However, several points require clarification or additional justification.

Major Comments

1. Missing citation for laser ablation evidence (Line 41): The claim at line 41 that laser ablation of astral MTs triggers spindle recoil is supported by citations to Bosveld et al. (2016) and Wu et al. (2024). However, the foundational laser ablation experiments demonstrating pulling forces on centrosomes by Stephan Grill and colleagues (Grill et al., 2001, Nature, and related work) should be cited here, as this is the primary evidence for cortical pulling forces in the field. The current citation list gives the impression this is a recent discovery rather than a well-established result.

We strongly agree that Grill et al. (2001) is the foundational work demonstrating the importance of cortical pulling forces on centrosomes. Accordingly, this sentence appears in the original manuscript: "Foundational evidence for the pulling model is derived from manipulations that disrupt the spindle midzone in the *C. elegans* embryo (Grill et al. 2001) (Figure 3C)." (Notably, these disruptions are not limited to laser ablations). We appreciate that this point should be raised earlier in the manuscript and have therefore rewritten a paragraph in the Introduction: "Two mechanisms have emerged as potential drivers of spindle orientation and positioning. The first of these involves dynein-dependent pulling on astral microtubules (Grill and Hyman 2005; Pecreaux et al. 2006; Kotak et al. 2012). Foundational evidence for pulling came from experiments in the *C. elegans* embryo; disruption of the spindle midzone in this system causes the spindle poles to

move rapidly apart, revealing outward-directed forces acting on the poles (Grill et al. 2001; Grill and Hyman 2005)."

Importantly, the original sentence referred to by the reviewer is one of three following the statement that "Multiple lines of experimental evidence identify astral MTs as the primary mediators of spindle orientation." We did not make a claim about cortical pulling here, but rather about the importance of astral MTs. We think that the citations are therefore appropriate, since the ablations in Grill *et al.* were performed on the spindle midzone, not the astral MTs.

2. Figure 1D and 1E: Dynamic instability of MTs not represented (Figure 1D, 1E): In Figure 1D, the pushing MT is depicted as a static filament, with no indication that it is in a growing (polymerizing) state. This is biologically important because pushing forces arise specifically from growing MT plus-ends encountering the cortex. A shrinking MT would not generate a pushing force. The authors explicitly state in the text (line 140-141) that pushing occurs when a "growing MT reaches the cortex," yet the figure does not convey this. The same issue applies to Figure 1E, the schematic should distinguish growing (pushing) from shrinking MTs more clearly in the context of force generation, consistent with the color-coding introduced later in Figure 2B. The authors should either add a polymerization indicator (e.g., growing tip markers) or an explicit label to clarify that this depicts a growing MT.

We agree with the reviewer's assessment. We have revised Figures 1D and 1E to clearly reflect the dynamic instability of the MTs. Specifically, we have added growing tip markers to represent the polymerizing state of the pushing MTs.

3. Model parameters: MT and force generator numbers require clearer justification. The authors use astral MT numbers scaled from *C. elegans* estimates (500-1000 per aster; Kozłowski et al., 2007) and a range of 40-400 force generators (FGs) citing Grill et al. (2003). The manuscript justifies its FG range of 40-400 citing Grill et al. (2003) for the estimate that ~50 FGs are required for spindle positioning. Pécéréaux et al. (2006) uses $N = 28$ motors per side of the AP axis (~56 total) in their model of anaphase oscillations in *C. elegans*, which is broadly consistent with the lower end of the range. However, both estimates come from anaphase dynamics in *C. elegans* specifically. The authors should clarify whether these numbers are appropriate starting points for the smaller *Drosophila* cells, where the range of 40-400 spans an order of magnitude without clear biological justification for the upper bound.

Firstly - We note a typographical error in the manuscript: the force generator (FG) range used in all simulations and figures is actually **20-200**, not 40-400. We apologize for the confusion and have corrected this in the revised manuscript.

Secondly - Since direct measurements for these parameters are currently unavailable in *Drosophila* follicle cells or zebrafish, we scaled our values from established *C. elegans* benchmarks as follows:

- *C. elegans* studies estimate that ~50 FGs are required for spindle positioning (Grill et al., 2003; Pécéréaux et al., 2006). Since *Drosophila* follicle cells are substantially smaller, we chose a conservative lower bound of 20 FGs. The upper bound of 200 FGs represents a fourfold excess over the *C. elegans* baseline; this was chosen not to model a specific biological condition, but to systematically explore how high FG density influences spindle orientation dynamics (as reported in Figure 2D).
- With regard to astral MT numbers, please refer to our response to comment #5.

We have updated the Methods section of the manuscript to explicitly include these biological rationales.

4. The spatial distribution of motors/FGs on the cortex is a critical parameter that shapes the outcome but is not systematically discussed. How sensitive are the results to the assumed spatial arrangement of FGs, particularly in the neuroblast and follicle cell conditions? The FG distribution is derived from fluorescence intensity profiles (Mud signal), but the mapping from fluorescence intensity to motor number is not validated. A sensitivity analysis varying FG distribution shape (e.g., width and peak position of the gradient) would substantially strengthen the modeling conclusions.

This is a good point. We agree that characterizing the systems' sensitivity to the spatial distribution of FGs is crucial, particularly given that mapping fluorescence intensity (Mud signal) to absolute motor numbers carries inherent uncertainties. We have therefore tested two additional distribution widths (15 degrees, or half as narrow, and 45 degrees, which is wider) for the follicle cell FG gradient (Supplemental Figure 7D). Under both conditions, all spindles orient correctly, and time to first passage was not significantly different from the 30° control (Kruskal-Wallis with Dunn's multiple comparisons). We did not alter the peak position of the gradient because our published microscopy results are clear that this is the center of the distribution in all cases examined.

We also undertook these simulations in simulated neuroblasts (Supplemental Figure 7E). All spindles oriented correctly regardless of distribution width; however, unlike follicle cells, FPT was not uniformly insensitive to spread — expanding the distribution from 30° to 45° significantly decreased FPT (Kruskal-Wallis with Dunn's multiple comparisons, adjusted $p = 0.0415$), indicating that neuroblast orientation speed is more sensitive to FG concentration than in follicle cells.

5. For *Drosophila* cells, the MT numbers used in simulations (50-400 in Figure 2D) are substantially below the *C. elegans* scaling estimate. The justification for this range in *Drosophila* specifically should be stated.

We thank the reviewer for raising this concern, and we have revised the text to state the rationale for the *Drosophila* astral MT numbers explicitly. Two points resolve the apparent discrepancy.

First, a unit conversion: throughout the manuscript, N_{MT} denotes the total number of astral MTs summed over both spindle poles, whereas the *C. elegans* value we quote (500-1000 per aster; Kozlowski et al. 2007) is per aster. Our *C. elegans* simulations accordingly use $N_{MT} = 1000$ (~500 per aster), consistent with that estimate. We had not stated this convention clearly and have now made it explicit where the value is first introduced.

Second, the *Drosophila* follicle cells and neuroblasts modeled here are substantially smaller in linear dimension than the *C. elegans* embryo (Supplemental Table 1), and astral MT number is expected to be correspondingly lower in smaller cells. We therefore used a reduced range for these cells. The precise total is not a sensitive parameter, because at any instant only a small, dynamically fluctuating subset of astral MTs is in contact with the cortex and mechanically engaged – consistent with direct measurements in human mitotic cells showing that each cortex-contacting astral MT is pulled by a single dynein motor (Anjur-Dietrich et al., 2024). The 50-400 range in Figure 2D was chosen to set a biologically plausible lower bound while systematically exploring how astral MT number affects spindle orientation dynamics; correct orientation is robust across the entire range (Figure 2D), so our conclusions do not depend on the specific value adopted. We have updated the model description accordingly.

6. Tension with Pécrciaux et al. (2016) on the role of pushing vs. pulling during *C. elegans* metaphase (Figure 3B): Pécrciaux et al. (2016) directly addresses spindle centering during metaphase in the *C. elegans* one-cell embryo. That paper independently estimates that ~1000 active force-generating elements underlie metaphase centering, which is consistent with the MT numbers used by Yeltokov et al. (500-1000 per aster from Kozlowski et al. 2007). However, Pécrciaux et al. (2016) reach a mechanistically important conclusion, they propose that the high precision of metaphase centering is accounted for quantitatively by MT pushing alone, and cortical pulling forces are argued to become dominant only during anaphase. This creates a direct tension with the Yeltokov model, which simultaneously incorporates both cortical pulling and pushing forces during the phase modelled in Figure 3B. This tension is not acknowledged or discussed anywhere in the manuscript. The authors cite Pécrciaux et al. (2016) only for simulation duration parameters (line 151), while overlooking its mechanistic conclusions that are directly relevant to their modeling choices. They should address this contradiction. Without this discussion, the paper's claim to provide a "unified framework" for *C. elegans* spindle dynamics is incomplete.

We thank the reviewer for raising this point, and we agree that the relationship between our model and the conclusions of Pécrciaux et al. (2016) should be made explicit. We now engage with its mechanistic findings rather than citing it only for timing parameters.

On revisiting that study, we would first note that its central conclusion concerns the maintenance (metaphase) phase specifically: the authors find that depletion of cortical force generators *improves* rather than diminishes centering, and conclude that the high precision of metaphase centering is best accounted for by length-dependent pushing from buckling astral MTs, with

cortical pulling not merely dispensable for but actively opposed to centering in this window (the authors note that cytoplasmic pulling cannot be excluded by their data).

We respectfully suggest that this represents a distinction in the quantity being modelled rather than a contradiction with our framework. The simulation the reviewer refers to (Figure 3B) concerns posterior *displacement* of the spindle – the asymmetric, off-center movement that Pécrcéaux et al. (2016) attribute to cortical dynein pulling – rather than maintenance-phase centering. Moreover, the pushing-based centering mechanism they propose depends specifically on the length-dependence of buckling forces, which is precisely the behavior captured by our Euler-buckling implementation. Our model is thus consistent with their proposal: length-dependent pushing supports centering, while asymmetric cortical pulling drives off-center positioning. We would add that the number of independent centering elements Pécrcéaux et al. (2016) infer (of order one thousand) corresponds to astral MTs rather than pulling force generators, and is consistent with the astral MT numbers we adopt (scaled from Kozłowski et al. 2007).

To make this explicit, we have adding the following to the Discussion: “Notably, Pécrcéaux et al. (2016) concluded that metaphase centering in this embryo is best explained by length-dependent pushing from buckling astral MTs rather than cortical pulling – the pushing mechanism our Euler-buckling term implements – with cortical pulling instead drives the subsequent asymmetric posterior displacement (Figure 3B)”.

7. Experimental validation of threshold behavior (Lines 216-225): The authors attempt to validate the model's predicted threshold behavior by depleting Mud using Traffic jam-GAL4 / UAS-mud-shRNA at varying temperatures (25°C, 27°C, 29°C). While the results are consistent with the model prediction, there is a significant confound, temperature affects not only GAL4 activity (and thus Mud levels / FG number) but also MT dynamics, including polymerization rate, catastrophe frequency, and rescue rate. This means the experiment is not cleanly changing a single parameter. The authors acknowledge none of this. At minimum, they should discuss this limitation explicitly and note that the temperature-dependent effects on MT dynamics could independently alter spindle behavior. Ideally, they would either cite evidence that MT dynamics are not significantly altered in the temperature range used in *Drosophila* follicle cells, or propose an alternative experimental approach (e.g., a partial loss-of-function allele of *mud*) that would more cleanly test the threshold prediction.

This is an interesting point. We have rewritten the relevant text to acknowledge temperature effects on MT dynamics. The reviewer also suggests the use of a partial loss-of-function *mud* allele. While such an approach would provide an independent test, interpretation is complicated by the fact that the extent of functional reduction is generally unknown; a lack of phenotype could therefore indicate either threshold behavior or just insufficient depletion. For this reason, we instead performed a genetic reduction using the protein-null allele *mud*^d and have included these results in the revised manuscript:

“We tested this prediction experimentally in the follicle epithelium by reducing Mud levels. Spindle orientation is normal in *mud*⁴/FM7 tissue, despite a 50% reduction in gene dosage (the *mud*⁴ allele is protein null) (Supplemental Figure 2). We also used the UAS–GAL4 system (Brand and Perrimon 1993) to express a previously validated UAS-mud-shRNA (Bergstralh et al. 2016). The follicle-epithelium driver Traffic jam-GAL4 (Olivieri et al. 2010) exhibits temperature-dependent activity, with stronger knockdown at 29°C and weaker knockdown at lower temperatures (Lovegrove et al. 2019; Neville et al. 2023). We therefore assessed spindle orientation in Traffic jam-GAL4 / UAS-mud-shRNA tissue at 25°C, 27°C, and 29°C. Misoriented divisions were observed at 29°C but not at the lower temperatures (Supplemental Figure 2). A potentially confounding consideration is that the change in temperature might also impact microtubule dynamics, though the range of temperatures used is narrow and physiological (Brankatschk et al. 2018). Together, these results are consistent with the model prediction that FG-mediated forces are robust to moderate weakening.”

8. Assumption of uniform FG distribution in zebrafish endothelial cells (Line 385): The authors acknowledge that the localization of cortical force generators in zebrafish ISV endothelial cells has not been determined (line 387). They then proceed to model the "simplest" case as a uniform distribution of FGs around the cortex. However, this choice of prior is not well-justified. The overwhelming evidence from other systems (*Drosophila* epithelia, neuroblasts, *C. elegans*, mammalian cells) is that FGs are asymmetrically distributed. Beginning from a uniform distribution therefore represents an assumption that is contrary to the general biology, not a neutral starting point. The authors should explicitly justify why uniform distribution is the appropriate null hypothesis here, rather than, for example, a distribution biased toward the proximal junction (which is where VE-Cadherin and ZO-1 are enriched, as the authors themselves show in Figure 6D). This choice has a material effect on the interpretation of Figure 6B–6C: the fact that uniform FG conditions fail to recapitulate spindle position may simply reflect that the uniform model is a poor biological prior rather than a meaningful mechanistic test.

This is a very thoughtful comment. We agree that a uniform distribution of force generators is not a biologically plausible description of the endothelial cortex, and we did not intend it as one. Accordingly, we used the term “simplest” instead of “most likely.” The reviewer correctly sees those terms as closer to equivalent than we meant. (Ockham would agree). We have revised the text to make our intended distinction clearer:

The uniform distribution is a *control*, not a hypothesis. Since pushing forces are symmetric and depend only on cell geometry, whereas directed off-center positioning requires an asymmetry in pulling, a uniform pulling field is the condition that removes all positional bias – allowing us to isolate what cell geometry, acting through pushing, can achieve on its own.

Two consequences follow from this, which we now make explicit in the text. First, our orientation conclusion does not depend on the assumed FG distribution: pushing alone, present in every

pushing-containing condition irrespective of FG arrangement, is sufficient to orient the spindle along the long axis (Figure 6B; Expanded View Figure 1B,C). The reviewer's concern about the uniform prior therefore does not affect the orientation result. Second, the comparison that carries mechanistic weight for positioning is not uniform-vs-nothing, but uniform-vs-junctional pulling, holding the density of FGs fixed and varying only their spatial arrangement. This is a controlled, single-variable manipulation: a symmetric arrangement cannot generate off-center positioning, whereas an arrangement biased toward the proximal cell-cell junction – where VE-Cadherin and ZO-1 are enriched (Figure 6D; Expanded View Figure 1D) - recovers both the correct angle and the observed off-center position (Figure 6B, C, F, G). This junction-biased distribution is precisely the alternative proposed by the reviewer, and it is this condition, not the uniform one, that we advance as the biological model.

The failure of the uniform condition to reproduce position is therefore informative not because uniform is a plausible prior, but because it establishes that positioning requires an asymmetry that symmetric pulling cannot supply. We have revised the text to frame the uniform condition explicitly as a control and to state that FG density is held constant across the uniform and junctional conditions, so that the contrast reflects spatial arrangement alone.

Minor Comments

Line 297-299: The authors correctly note that the apparent trend in the pushing-only ensemble average is an artifact of averaging stochastic paths. This point could be made more visually obvious by showing individual trajectories (which the authors say are in Supplemental Figure 4A) directly in the main text figure, or at least referencing this panel more prominently here.

We thank the reviewer for this suggestion. In the revised manuscript we have revised the manuscript text to reference Supplemental Figure 4A more prominently at this point, directing readers explicitly to the individual simulation trajectories. "Supplemental Figure 4A shows all individual simulation runs, confirming that pushing-only trajectories explore angle space stochastically without converging toward the correct orientation."

Line 302-304: The authors note that spindle alignment occurs faster in their model than in chick neuroepithelial data (Peyre et al., 2011) and attribute this to underestimation of cytoplasmic drag or overestimation of cortical forces. This discrepancy deserves more attention, are the timescales qualitatively or quantitatively different? A brief quantitative comparison would clarify whether this is a minor calibration issue or a more substantive model limitation.

We thank the reviewer for raising this point. We have performed a quantitative comparison and find that FPTs in our model are parameter-dependent, spanning a range that includes the ~300 second timescale reported in Peyre et al. (2011). Specifically, our heatmap analysis of FPT as a function of pulling and pushing strength shows that parameter combinations within the physiologically plausible range produce FPTs on the order of 300 seconds, quantitatively

consistent with the ~5 minute orientation timescale observed in chick neuroepithelial cells. We have revised the manuscript accordingly

Line 380: "<45" and "<30" should include degree symbols for consistency with the rest of the manuscript.

Good catch, thanks! We have added those degree symbols.

Line 382: "~3.5m" should read "~3.5 μm " (missing micron symbol).

Thanks. We have replaced "~3.5m" with "~3.5 μm ".

Figure 4 legend: The legend states "Boxplots as in (C)" for panel E, but panel C is a simulation snapshot, not a boxplot. This appears to be a mislabeling and should reference panel D instead.

The reference "Boxplots as in (C)" is indeed incorrect. We have corrected this to reference panel D in the revised legend.

Figure 5A' (microscopy panel): The time stamp "231" appears twice in the image sequence (frames 6 and 7 as labelled), suggesting a labeling error in the figure. This should be checked and corrected.

The reviewer is right! We've corrected the error.

The simulation of *C. elegans* spindle posterior displacement (Figure 3B) does not incorporate spindle elongation, which is known to be critical for accurate posterior positioning in this system. While the authors note this limitation, they should more explicitly discuss what the model can and cannot conclude about the *C. elegans* positioning mechanism given this omission.

We agree and have expanded both the Results and the Limitations to state explicitly what our model can and cannot conclude about *C. elegans* positioning given this omission:

"Since we treat the spindle as a rigid body of fixed length, this simulation isolates the translational contribution of asymmetric cortical pulling from that of anaphase-B spindle elongation, which is known to be critical for accurate posterior positioning in this system (Tsou et al. 2002). Within this scope, the results show that asymmetry in cortical pulling between anterior and posterior domains is sufficient to displace the spindle center posteriorly while maintaining A-P alignment (Figure 3B'-B", Supplemental Figure 3B, Movie S4), capturing the direction and qualitative character of off-center positioning. However, the absolute positions of the two poles – and hence the cleavage plane – are set *in vivo* by this displacement together with elongation. Our model thus reproduces

the net posterior shift of the spindle center but not the final pole positions, the pronounced differential pole movement seen in the embryo, or the precise division-plane location.”

The Limitations section (lines 433-450) is commendably thorough, but the omission of cytoplasmic pulling forces (line 448) is listed as an afterthought. Given that cytoplasmic pulling has been specifically shown to contribute to centering in *C. elegans* (Kimura and Kimura 2011; Kimura and Onami 2005), this limitation is particularly relevant for the *C. elegans* section and should be discussed there rather than only in the Limitations paragraph.

We have added a more thorough explanation of our reasoning to the introduction:

“Dynein-dependent pulling of astral MTs has also been proposed to arise within the cytoplasm rather than at the cortex. This is suggested by observations in large embryonic cells, including *Xenopus*, zebrafish, and sea urchin embryos, in which asters can begin to move before astral MTs contact the cortex (Wühr et al. 2010; Tanimoto et al. 2016, 2018). However, there is less support for the possibility that cytoplasmic forces contribute to spindle positioning and orientation in smaller cells, in which astral microtubules are expected to contact the cortex regularly. Although cytoplasmic pulling has been suggested to explain centrosome migration in the one-cell *C. elegans* embryo (A. Kimura and Onami 2005), subsequent work found no evidence for such a mechanism (Wu et al. 2024). The mechanistic basis for cytoplasmic pulling in this cell also remains unclear. One proposal is that dynein associated with intracellular organelles generates length-dependent pulling forces on astral MTs (K. Kimura and Kimura 2011), but large-scale imaging in *Xenopus* egg extracts showed that MTs and cytoplasmic organelles largely co-migrate rather than moving past one another (Pelletier et al. 2020).”

The third reviewer raised a similar issue (their Comment #2) and was explicitly concerned that cytoplasmic pulling could fulfill the “geometric sensor” function that our model ascribes to pushing. In light of that concern, we implemented cytoplasmic pulling as an alternative to pushing in irregularly-shaped Zebrafish vascular endothelial cells. Our findings are included in the revised manuscript and detailed in our response to Reviewer #3.

Referee #2 (Report for Author)

Yeltokov *et al.* have constructed a computational model of spindle orientation and positioning involving microtubule pushing against the cell cortex and pulling with the help of cortical force generators such as the dynein molecular motors. They apply this model to explain spindle orientation and positioning in a range of asymmetrically dividing cells, from classical model systems such as *Drosophila* S2 cells and the first division of a *C. elegans* embryo, to less well studied and more heterogeneous Zebrafish endothelial cells. In the latter model, the authors use a combination of image segmentation and computational simulations to compare the experimental time-lapse movies of spindle orientation establishment with the results of a computational model provided the cell shape as the input. The authors then vary parameters of their model to analyse

the contributions of microtubule pushing, cortical pulling, and the spatial distribution of the cortical force generators to the observed similarity between experimental and simulated spindle orientations. The authors conclude that in this Zebrafish system, microtubule pushing forces mainly contribute to spindle orientation, while asymmetrical pulling forces mainly contribute to spindle positioning.

I found the paper written in an accessible manner for people without mathematics/physics background like myself, with clear figures. The strongest part of the reported data are the side-by-side comparisons between experiments and simulations for dynamically reshaping, irregular cells undergoing asymmetric division (reported in Fig 5-6) - this is certainly novel and interesting. I found other parts of the paper less convincing/interesting, and I have several suggestions to improve the strength of the main arguments.

1. For comparisons between experiments and simulations in Fig 5-6, the authors report only the difference between the experiment and the simulation for both the spindle angle and the spindle position. It is unclear what is an acceptable $\Delta(\text{obs-model})$ value? Perhaps better would be to directly report the distributions of experimental values, and separately for simulation values for each condition tested, such as in Fig 6FG. This will be important for the readers to get a better understanding of inherent variability of spindle orientations and positions, and of the model's performance in reproducing these experimental values. The authors could then compare the simulations with the experimental observations directly - as opposed to the currently reported comparisons of $\Delta(\text{obs-model})$ for different model configurations. Additionally, are the results of the simulation robust over several runs with the same shape of the cell? Reporting this directly, in my opinion, would be more informative than the current presentation where only the $\Delta(\text{obs-model})$ values are reported, not the absolute values per run/per cell.

Unfortunately, plotting absolute values across cells is not straightforward: the 12 cells differ in size and shape and the degree of spindle displacement varies without a clear trend, meaning that raw position values are not directly comparable across cells. We are therefore concerned that presenting the raw data in the main figure would obfuscate rather than clarify the results. However, we certainly appreciate the reviewer's point and in response we have changed Supplemental Figure 5 so that it shows these "raw" numbers, allowing the reader to evaluate them.

2. The authors make an attempt to produce a unified model with a single parameter set that they then apply to a range of experimental model systems. I wonder if they checked how sensitive are their results to microtubule growth/shortening speeds and frequencies of rescue/catastrophe? Currently, the four parameters describing microtubule dynamic instability are taken from two experimental reports - one on BS-C-1 cells from the African green monkey (Burakov JCB 2003), the other on *Drosophila* S2 cells (Rogers JCB 2002), and one theoretical study on *C. elegans* (Kimura & Onami Dev Cell 2005, which in turn derives these parameters from other experimental studies on a range of models from newt lung cells to *Xenopus* extracts). However, these dynamic parameters can be different in different experimental systems. In *C. elegans* the microtubule

dynamics can be quite variable and the values reported, for example in Lacroix Dev Cell 2018, differ by several fold from the values used in the model. Are these parameters known for Zebrafish endothelial cells?

We thank the reviewer for this important point. We agree that MT dynamic parameters can vary substantially across cell types and organisms, and that the values we used are drawn from different experimental systems that may not perfectly match our model organisms. To address this directly, we performed a systematic sensitivity analysis by scaling growth and shrinkage rates together — preserving their ratio — using multiplicative factors of 0.1, 0.25, 0.5, 1 (control), 2, 4, and 10× applied to control values (Supplemental Figure 7B). All spindles oriented correctly across the full range tested, and FPT was not significantly different from control across most conditions (Kruskal-Wallis with Dunn's multiple comparisons, adjusted $p = 0.0343$), with a slowdown emerging only under substantially suppressed turnover. We performed the same analysis for catastrophe and rescue rates, scaling them together over the same range of multiplicative factors (Supplemental Figure 7C). Again, all spindles oriented correctly and FPT was not significantly different from control across the full range tested.

Regarding MT dynamic parameters in zebrafish endothelial cells specifically — to our knowledge these have not been directly measured, and we acknowledge this as a limitation.

3. I found the title slightly misleading. At the end of their Results section the authors conclude that "an asymmetric pulling force ... required to correctly position the spindle", based on results reported in Fig 6. This is a little hard for me to reconcile with "beyond pulling" in the title?

We appreciate the reviewer's point and have changed the title to "Microtubule pushing and cortical pulling cooperate to orient and position the mitotic spindle".

4. There are quite a few computational models reported in the literature that consider the contributions of both microtubule pulling and pushing forces in a variety of systems - some the authors have cited (Letort MBoC 2016, Kozlowski Cell 2007), some not (such as Pavin New J Phys 2012 and related papers). Perhaps the authors may wish to expand their quite compact Discussion to place their new model and results in the context of these previously published models - this will help a broader audience to appreciate what is novel in the current work. Since the authors consider their spindle to be simply a passive object being positioned, from the modelling point of view it is quite similar to a centrosome/MTOC and so the authors could use models that consider aster positioning for comparison.

We have substantially expanded the Discussion to situate our model within the broader computational literature. We now compare our framework with prior models of both cortical pushing and pulling, including the aster-positioning models the reviewer highlighted (Pavin et al. 2012; Ma et al. 2014), alongside Kimura and Onami (2007), Farhadifar et al. (2020), Letort et al. (2016) and Kozlowski et al. (2007). We also take the reviewer's point that, because we treat the

spindle as a passive object, its positioning is formally analogous to that of a centrosome/MTOC; we now make this correspondence explicit, so that these aster-positioning models form the natural comparison class. The expanded Discussion articulates what our framework adds relative to these - the systematic separation of pushing-only, pulling-only and combined regimes within a single, consistently parameterized model, applied to non-rounding cells with measured, time-evolving shapes (see also our response to Reviewer 3, comment 3).

Minor comments:

5. In experiments reported in Supplementary Fig 2, are there any force generators left at the cortex, or is it known how strongly are they depleted compared to normal levels? What would be the outcome of the simulations reported in Fig2D/D' in a condition with zero FG?

We do not have a precise measurement of how much Mud remains at the cortex in these experiments. We have an anti-Mud antibody, but it is unfortunately not reliable/sensitive enough for us to make confident cross-sample comparisons of cortical signal intensity. A weaker signal could reflect either genuine depletion or reagent variability. Robust immunodetection of cortical Mud/NuMA is a recognized difficulty in the field. Our interpretation does not, however, require the absolute residual level to be known. The model predicts a *threshold* number of functional FGs below which orientation fails. The misorientation we observe (Supplementary Figure 2) is consistent with the perturbation crossing this threshold.

A condition with zero FGs corresponds to the pushing-only regime. Since the follicle cells and neuroblasts are modeled as circular, pushing forces in this geometry carry no long-axis bias – unlike in the elongated endothelial cells, where cell shape biases pushing – and so provide no directional cue toward the correct orientation. Spindle-angle trajectories in the zero-FG condition are therefore dominated by chance rather than directed rotation: the success rate falls to near the level expected from random orientation, and any first passage time reflects chance entry into the target range rather than a meaningful orientation timescale. For this reason, we do not include the zero-FG condition as an interpretable entry in the Figure 2D/D' heatmap.

6. Results reported in Fig4 suggest that the "pulling only" model works reasonably well in allowing the spindle to eventually reach the normal orientation. The "pushing only" model, on the other hand, performs poorly. To me this result seems to contradict the data in Fig 2D/D' where the model with a decreasing number of FGs works increasingly bad, while the remaining pushing forces seem to be sufficient? Is there something I miss in these interpretations?

We regret this confusion, which appears to stem from lack of clarity about the conditions used in each figure. The simulations presented in Figure 2 are run exclusively in the combined pulling and pushing condition; reducing FG number weakens pulling forces but never eliminates them. Even at the lowest FG numbers shown, residual pulling forces remain present and continue to drive orientation, but less efficiently. This is not equivalent to the pushing-only condition in Figure 4, where cortical pulling is entirely absent. The two figures are in fact consistent: pulling is the

primary driver of orientation, and its partial reduction slows but does not abolish alignment, while its complete removal causes orientation to fail. In the revised manuscript we have added an explicit statement at the start of the validation section clarifying that all simulations there use the combined condition and note that the dissection of individual force contributions is addressed subsequently in Figure 4: "We first validated the model under the combined regime, in which both pushing and pulling mechanisms are active simultaneously, because this setting tests the full capacity of the model. The effect of disabling either mechanism is examined separately in Figure 4."

Referee #3 (Report for Author)

Yeltokov et al. developed a 2D agent-based computational model integrating cortical dynein-mediated pulling forces and MT polymerization-based pushing forces to study mitotic spindle orientation and positioning (Fig. 1). The central claim is that this constitutes a "generalizable" framework applicable across diverse cell types, in contrast to prior cell-type-specific models. The model is validated against established experimental observations in *Drosophila* follicular epithelial cells and neuroblasts (Fig. 2) and in the *C. elegans* embryo (Fig.3), and is then applied to zebrafish angiogenic endothelial tip cells (Figs. 5, 6). The principal scientific conclusions are that MT pushing forces act as geometric sensors of cell shape, prevent spindle stalling by buffering poles away from the cortex, and promote spindle dynamicity-roles proposed to be complementary to, rather than redundant with, cortical pulling.

The ambition to apply a single unified modeling framework across multiple organisms and cell types is commendable and represents a valuable direction for the field. However, for such a framework to support the conclusions drawn here, the model must be adequate for the specific claims being made. As detailed below, I have serious concerns that the current model lacks key components necessary to substantiate the claim of generalizability and, as a consequence, the scientific conclusions regarding the role of MT pushing forces cannot be adequately evaluated.

Major Concerns

#1: The 2D formulation undermines the claim of generalizability: The model operates in a 2D midplane cross-section, in which the cortex is represented as a 1D boundary. In a real 3D cell, the cortex is a 2D surface, and the number, angular distribution, and force balance of MT-cortex interactions are qualitatively different. This distinction is especially consequential here, where cortical interactions are the central subject of inquiry. The authors' rationale for using 2D-that experimental data are often available only in 2D-does not hold, since 3D simulations can be compared with 2D data via projection.

Importantly, 3D models of comparable complexity to the present work already exist in the cortical pulling literature. For example, Kimura & Onami (J Cell Biol 179, 1347-1354, 2007) and Farhadifar

et al. (eLife 9, e55877, 2020)-neither of which is cited in the present manuscript-demonstrate that 3D formulations are tractable at a similar level of modeling complexity. The current 2D framework therefore does not represent a technical advance in this respect.

I consider 3D validation of the principal conclusions, particularly those concerning cell-shape-dependent pushing forces and the zebrafish endothelial cell application, to be necessary before the generalizable nature of this model can be accepted.

We thank the reviewer for this substantive critique, which has prompted us to state the scope and justification of our modeling choice far more carefully. We have made four changes in response.

First, we accept the core limitation and no longer claim otherwise. We have removed all assertions of a general “generalizable” framework and now describe the model explicitly as a 2D midplane formulation. We agree that in a real 3D cell the cortex is a 2D surface and that the number and angular distribution of MT-cortex interactions differ from their midplane counterparts; we state this plainly in both the Results and the Limitations.

Second, we have clarified which of our conclusions depend on dimensionality and which do not - a distinction we should have drawn in the original submission. Our central mechanistic claims are that pushing forces are length-dependent and therefore encode proximity to the cortex, that this geometric readout can orient the spindle along the long axis of an elongated cell, and that an asymmetry in cortical pulling is separately required for off-center positioning. None of these rests on the cortex being 1D vs 2D: the length-dependence of buckling forces, and the requirement for a pulling asymmetry to break positional symmetry, hold identically in 3D. What a 2D midplane cannot capture is the quantitative force balance – out-of-plane MTs and FGs contribute off-axis forces and torques – and we note that our quantitative predictions (first-passage times, absolute angles, and positions) should be read as within-plane estimates rather than fully resolved 3D values.

Third, we set out a positive justification for the 2D choice specific to these claims, rather than resting on data availability alone. For the elongated zebrafish cells that motivate the study, the biologically relevant question is how in-plane cell shape biases spindle orientation and position; the 2D midplane is the plane in which the spindle is imaged and in which its long-axis alignment and off-center displacement are defined. Restricting to this plane isolates the role of boundary curvature and pulling asymmetry from out-of-plane cortical anisotropy, which would enter a 3D model as unconstrained free parameters. A 2D formulation therefore permits high-throughput, per-cell simulation across our full experimental dataset at a level of parameter identifiability that a 3D model of the same cells could not currently support.

Finally, we agree that our framing implied a technical advance over existing 3D models, which was not our intention and which we have removed. We now cite Kimura & Onami (2007) and Farhadifar et al. (2020) in the Discussion as demonstrations that 3D formulations are tractable at

comparable complexity, and we position our contribution not as a dimensional advance but as the systematic separation of pushing-only, pulling only, and combined regimes within a single framework, applied to non-rounding cells with experimentally measured, time-evolving shapes – a regime these prior models did not address.

#2: Exclusion of cytoplasmic pulling forces undermines the primary scientific conclusions

The authors exclude cytoplasmic pulling forces-length-dependent forces exerted by cytoplasmic dynein throughout the cell volume-on the grounds that cortical interactions dominate spindle behavior. This exclusion is not adequately justified. It is well established that cytoplasmic pulling forces can encode cell shape information and direct spindle orientation (e.g., Minc et al. Cell 144, 414-426, 2011; Kimura & Onami 2007), overlapping substantially with the "geometric sensor" function attributed to MT pushing in this manuscript. A model that excludes cytoplasmic pulling cannot distinguish between the contributions of pushing and cytoplasmic pulling, and therefore cannot establish that the effects attributed to pushing are not in fact mediated by cytoplasmic pulling. Demonstrating that the proposed role of MT pushing cannot be fulfilled by cytoplasmic pulling forces is essential to the central argument of this paper, and this comparison is entirely absent. A model claiming generalizability should incorporate both cortical and cytoplasmic force-generating mechanisms.

The reviewer makes a thoughtful and careful point. We chose not to include cytoplasmic-pulling in the previous manuscript for the following reasons:

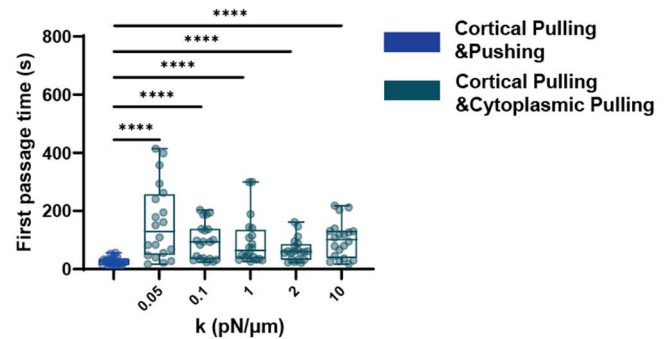
- 1) Cytoplasmic pulling in the one-cell *C. elegans* embryo is not well supported. The Kimura and Onami 2007 paper cited by the reviewer appears to be a typo. We assume the intended reference is Kimura and Onami 2005. While that paper shows that pulling is important for centrosome migration in the one-cell *C. elegans* embryo, it does not experimentally distinguish the source of the pulling (cytoplasmic vs. cortical). Subsequent work cited in our manuscript (Wu *et al.* Nature Physics 2024) shows that “cortical pulling forces drive pronuclear migration and rotation as well as spindle centering, elongation and oscillations in *C. elegans* embryos, with no discernible contribution from MT pushing or cytoplasmic pulling forces.” (Emphasis ours). Thus, while Kimura and Onami 2005 show that pulling is important for centrosome migration, the source of this pulling is not cytoplasmic but cortical. (Importantly, our *C. elegans* embryo simulations (Figure 3) include length-dependent MT pushing. We make no claim that pushing is necessary in this cell – only that the pushing we’ve implemented is consistent with experimental observation - and our results are therefore in agreement with Wu *et al.*).
- 2) A mechanistic basis for cytoplasmic pulling is not clear. Computational modeling in the Minc *et al.* study cited by the reviewer is consistent with the possibility that cytoplasmic pulling mediates geometric sensing, but they are A) careful not to claim this as an experimentally-verified conclusion and B) pushing is excluded from their model. One study (Kimura and Kimura PNAS 2011) proposes that cytoplasmic pulling could work by tugging on organelles. However, a subsequent study tested this model using egg extracts

(Pelletier *et al.* 2020) and found that at large scale, microtubules do not move past static organelles. Instead, they comigrate.

We now describe these findings in the introduction. The relevant text is quoted in our response to Reviewer #1's final comment.

In a more direct response to this specific concern, we have also extended the model to include cytoplasmic pulling implemented as a force that increases with MT length and expanded the Methods section accordingly. Since no experimental measurement of this coefficient exists for our model systems, we tested a range of values ($k = 0.05, 0.1, 1, 2, 10, 20$ pN/ μ m).

The equivalent parameter range in Kimura and Onami (2005) spans approximately 0.01–0.75 pN/ μ m. In our follicle cell simulations, values in this range produced frequent spindle stalling, indicating that this force is insufficient to counterbalance cortical pulling. Stable orientation without stalling required $k = 10$ pN/ μ m, while $k = 20$ pN/ μ m produced excessive oscillation inconsistent with biological observations. At $k = 20$, spindles exhibited excessive oscillation and erratic movement inconsistent with biological observations. Comparing the $k = 10$ condition to our cortical pushing + cortical pulling control, first passage time analysis shows that cortical pushing still produces faster spindle orientation than cytoplasmic pulling at matched cortical pulling strengths, suggesting that cortical pushing provides a more efficient orienting mechanism (Mann-Whitney test, $N=20$). We have chosen to show those results here but have not included them in the manuscript.



As the reviewer points out, cytoplasmic pulling is indeed a potential mechanism for shape sensing. As this should be especially relevant in zebrafish ISV cells, which have irregular shapes, we modeled cytoplasmic-pulling in these cells. We find that while spindles can orient at $k = 10$ pN/ μ m, the strength of cytoplasmic-pulling prevents off-centered spindle positioning. At $k = 1$ pN/ μ m, spindle positioning is correct but orientation fails. These results are incorporated into the revised manuscript:

“Our results suggest that spindle orientation in angiogenic endothelial cells is dictated primarily by cell geometry. If so, an independent mechanism that encodes cell geometry should produce a similar outcome. While extensive experimental evidence supports microtubule pushing (Dogterom and Yurke 1997; Holy *et al.* 1997; Tran *et al.* 2001; Janson *et al.* 2003; Daga *et al.* 2006; Laan *et al.* 2012; Sulerud *et al.* 2020; Cueff *et al.* 2024), length-dependent cytoplasm-based microtubule pulling has also been proposed to translate cell shape into spindle orientation (A. Kimura and Onami 2005; Minc *et al.* 2011). We therefore asked whether this independent

mechanism could orient the spindle in irregularly shaped angiogenic endothelial cells. We implemented cytoplasmic pulling forces following the length-dependent formulation $F_{cyto} = k \times L$, where k is a scaling coefficient. All microtubules are subject to cytoplasmic pulling and pushing is excluded. We tested three values for k : 0.05, 1, and 10 pN/ μ m. The first two are consistent with a range of values used previously and considered biologically relevant (A. Kimura and Onami 2005). Spindle orientation along the cell long axis was achieved at all three values, though it was marginally less accurate at 10 pN/ μ m (Supplemental Figure 6). These results support the idea that spindle orientation in angiogenic endothelial cells can be dictated by cell geometry without the need for force generators pulling from the cortex. Importantly, we do not consider these results to be evidence for cytoplasmic pulling in our system. Angiogenic endothelial cells are not large, and their width ($\sim 10 \mu$ m) is comparable to the length of the spindle. Astral microtubules are therefore expected to contact the cortex frequently, and we therefore expect cortical interactions to predominate spindle orientation and positioning in these cells.”

Minor Concerns

#3: Advantages over existing models are not articulated

The authors position their model as a generalizable alternative to prior cell-type-specific models, but do not explain what the present framework offers beyond existing approaches. Many prior models are themselves extensible in principle, and it is not clear what technical or conceptual innovations enable the claimed generalizability here. The authors should explicitly compare their framework with the most relevant prior models and articulate where and why the present approach offers advantages. This is particularly important given that the current model is, in some respects-dimensionality, force mechanisms included-less complete than existing formulations.

We thank the reviewer, and we accept that we asserted advantages over prior models without substantiating them. Reviewer #2 raised a closely related point (their comment 4), and we address both here.

First, we have removed the framing of our model as a "generalizable" alternative to prior cell-type-specific models (see also our response to Major Concern #1). Our model is, in several respects, deliberately less complete than existing formulations - it operates in a 2D midplane and considers only cortical pulling and pushing - and we no longer present completeness or generality as the contribution.

Second, we have expanded the Discussion to compare our framework explicitly with the most relevant prior models. Analytical and agent-based models have established the force mechanisms underlying centering and orientation (e.g. Howard 2006; Kozlowski et al. 2007; Ma et al. 2014), including three-dimensional implementations (Kimura and Onami 2007; Farhadifar et al. 2020) and models spanning varying cell geometries (Letort et al. 2016). Against this background, we articulate our contribution as resting on two features not typically combined in prior work: (i) the

systematic isolation and comparison of pushing-only, pulling-only, and combined force regimes within a single, consistently parameterized model, allowing the role of pushing to be dissected rather than assumed; and (ii) application to experimentally measured, time-evolving shapes of a non-rounding cell type (angiogenic endothelial tip cells), a setting in which we find that spindle orientation and positioning can be decoupled. We are careful not to claim that dynamic or non-standard geometries have never been modeled - Letort et al. (2016), for example, considered varying shapes - but rather that combining controlled force-regime dissection with measured, evolving geometries of this cell type yields the specific mechanistic separation we report.

#4: Sensitivity of conclusions to MT length distribution

Pushing forces in the model are inversely proportional to the square of MT length, making the relative contributions of pushing and pulling-and thus the principal conclusions-potentially sensitive to the assumed MT length distribution. While MT number and FG number are varied systematically, MT length distribution is not. The authors should identify which conclusions are robust across a range of length distributions and which are not. Given the strong length dependence of the pushing force, this analysis is necessary to assess the reliability of the quantitative predictions.

We thank the reviewer for this constructive critique. We agree that given the inverse-square dependence of pushing forces on MT length, the assumed MT length distribution is a critical factor that could influence the balance between pushing and pulling mechanisms. To test the reliability of our predictions, we performed a sensitivity analysis by systematically varying the initial MT length distribution (Supplemental Figure 7A). MT initial lengths in our model are drawn from a gamma distribution (shape, scale), chosen for its physiological plausibility as a right-skewed, zero-bounded length distribution; the control condition (shape = 1, scale = 1) is exponential with mean ~10 μm . We tested distributions with the same mean but altered variance (shape = 4, scale = 0.25, reduced variance; shape = 0.5, scale = 2.0, increased variance with a heavier tail), a distribution weighted toward shorter MTs (shape = 1, scale = 0.5, mean ~5 μm), a distribution weighted toward longer MTs (shape = 1, scale = 1.5, mean ~15 μm), and constant initial-length conditions (all MTs fixed at either 10 μm or 1 μm) in place of the gamma distribution. All spindles oriented correctly across all conditions tested, and FPT was not significantly different from control, with the exception of the 1 μm constant-length condition, in which orientation took significantly longer — as expected, since MTs must grow sufficiently to engage the cortex and contribute to force generation (Kruskal-Wallis with Dunn's multiple comparisons). These results indicate that the principal conclusions of the model are robust to the assumed initial MT length distribution.

#5: Accuracy of descriptions of prior work

In the *C. elegans* sections, I identified at least one statement that appears inaccurate. At L.282-284, the authors state that spindle orientation "generally fails" after disruption of the canonical pulling machinery. This overstates the phenotype: it is well documented that spindle orientation is not entirely abrogated under these conditions in *C. elegans*, and the relationship is more nuanced than implied. I raise this as an example rather than an isolated concern-given the breadth of

systems covered in this manuscript, I would ask the authors to carefully review all descriptions of prior experimental and computational work for accuracy, and to ensure that the novelty of the present contributions is clearly delineated from what has already been established.

Thanks for pointing this out. We have both corrected the specific statement identified and conducted a systematic review of our descriptions of prior work. On the specific point, the reviewer is correct that "generally fails" overstates the phenotype, and we have revised the sentence accordingly. Prompted by this comment, we reviewed our descriptions of prior experimental and computational work throughout the manuscript and made the following corrections, several of which also address other reviewer comments: (i) we clarified the scope of the prior cortical-pulling manipulation experiments discussed in the Introduction (Reviewer 1, comment 1); (ii) we now describe the conclusions of Pécrciaux et al. (2016) accurately, noting that they attribute metaphase centering to length-dependent pushing rather than to cortical pulling (Reviewer 1, comment 6); (iii) we cite and delineate our work from existing three-dimensional positioning models (Kimura and Onami 2007; Farhadifar et al. 2020), clarifying that our contribution is not a change in dimensionality but the systematic separation of pushing-only, pulling-only, and combined regimes in non-rounding cells with time-evolving, experimentally measured shapes (this reviewer's comments 1 and 3); and (iv) we reframed the uniform force-generator distribution as a control rather than a biological model (Reviewer 1, comment 8). Together, these changes improve the accuracy of our prior-work descriptions and more clearly delineate the novelty of the present contribution.

Dear Dan and Holly,

Thank you for submitting a revised version of your manuscript to The EMBO Journal. I sincerely apologise for the delay in the assessment process due to summer holidays and the resulting backlog. Your study has now been seen by all original referees, and their comments are included below.

While reviewers #1 and #2 are generally satisfied with the revision, reviewer #3 finds that some further improvements are needed, such as reorganisation of the article and textual editing to emphasise the relevance of the model for cells with complex geometries, as well as to increase the transparency of the analysis and discussion in the context of previous literature.

Therefore, I would like to invite you to address these remaining requests by reviewer #3 in a final version of the manuscript. Additionally, there are a few editorial and formatting points that need to be addressed before I can extend the official acceptance of the manuscript:

1. Please note that it is The EMBO Journal policy for the transcript of the editorial process (containing referee reports and your response letters) to be published as an online supplement to each paper. If you should prefer removal of any referee-only figures included in the point-by-point response(s), e.g. because they may still be used for future publication or because they have been reproduced from published work by others, please do let us know immediately via response email. More information is available here: https://www.embopress.org/transparent-process#Review_Process
2. In the "Data Availability" section, please state: "This study includes no data deposited in external repositories". Further information on the format can be found at <https://link.springer.com/partners/embo-press/editorial-policies#Data%20availability%20statement>.
3. Please ensure that the figures are cited in sequential order. Currently, Fig 2B-C is called out before Fig 4 in the manuscript text.
4. Please correct the nomenclature of the expanded view figures to "Figure EV1" and "Figure EV2".
5. For the Appendix items, please correct the nomenclature to "Appendix Table S1" and "Appendix Figure S1" etc. Please remove movie legends from the Appendix file.
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Please let me know if you have any questions regarding any of these points. You can use the link below to upload the revised files.

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal. I look forward to receiving the final version.

With best wishes,

Ieva

Ieva Gailite, PhD
Senior Scientific Editor
The EMBO Journal
Meyerohofstrasse 1
69117 Heidelberg
i.gailite@embojournal.org

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 (8th Dec 2026). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

<https://emboj.msubmit.net/cgi-bin/main.plex>

Referee #1:

This revised version is now significantly improved and all my concerns are sufficiently addressed. Reading through the comments of reviewers 2 and 3 it seems to me that those comments were addressed as well. I have no further points that would need to be addressed.

The current findings and conclusions are of medium significance and high priority for the wider readership of EMBO Journal. This assessment is based on the fact that multiple groups and models have already investigated the role of cortical pushing and pulling forces during spindle positioning. Thus, the manuscript does not provide novel information per se but rather extends and confirms existing models.

Referee #2:

The authors addressed all concerns I had listed previously. No further suggestions.

Referee #3:

The two principal concerns I raised in the first round-the overclaiming of a "generalizable" model and the exclusion of cytoplasmic pulling forces-have been addressed in a manner I find broadly satisfactory. Withdrawing the generalizability claim and repositioning the model as a tool suited to the analysis of complex cell geometries such as those of vascular endothelial cells is an appropriate response, and I accept this reframing. The addition of Supplemental Figure S6 exploring cytoplasmic pulling is also appreciated.

That said, the manuscript still carries significant structural weight from its original framing, and I believe a further round of revision is needed before it can be accepted.

Comment 1: The manuscript's focus and structure should be realigned with the revised framing

Now that the authors have repositioned this as a study of spindle orientation and positioning in complex cell geometries-with the zebrafish vascular endothelial cell as the central application-the structure of the manuscript should reflect this. At present, the paper devotes considerable space (currently spanning approximately four pages of the Results) to simulations of *Drosophila* and *C. elegans*, systems with simple, well-characterized geometries for which three-dimensional models already exist and have been extensively validated. This creates an imbalance in emphasis that works against the authors' revised narrative.

1.1 Refocus the manuscript throughout

The research focus-understanding spindle orientation and positioning in cells with complex geometries-should be stated clearly and consistently from the Introduction through to the Discussion.

For example, rather than the current section heading "A novel computational model for spindle orientation," a heading such as "A 2D computational model for spindle orientation and positioning applicable to diverse cell geometries" would be more informative and more accurately reflect the revised framing.

1.2 Substantially condense the treatment of Figures 2 and 3

The simulations of *Drosophila* follicular epithelial cells, neuroblasts, and the *C. elegans* embryo serve a legitimate purpose as validation of the model against well-established experimental observations. However, given the revised focus of the paper, this material should be condensed substantially so that the manuscript reaches the zebrafish data more efficiently. I would suggest treating these results as model validation and reducing their coverage in the main text accordingly. Moving Figures 2 and 3 to the Supplemental section is one option the authors may wish to consider, though I do not require this; what I do require is that the current level of detail in the main text be significantly reduced.

1.3 Consider repositioning Figure 4

Figure 4 presents a detailed mechanistic analysis of how MT pushing prevents spindle stalling and promotes dynamicity in follicle cells. In the current structure, this analysis precedes the zebrafish data (Figures 5 and 6) that motivates it. I would suggest that Figure 4 could be positioned after Figures 5 and 6, so that the mechanistic dissection follows naturally from the observation in Figure 6 that pushing forces are needed to prevent over-correction by junctional pulling. This reordering would strengthen the logical flow of the paper. I offer this as a suggestion rather than a requirement, as the authors are in the best position to judge the overall narrative.

1.4 Tone down quantitative comparisons between simulation and experiment

Because the model is two-dimensional and the parameter values used were originally derived mostly in the context of three-dimensional models, precise quantitative agreement between simulation outputs and experimental measurements cannot be meaningfully claimed. I would ask the authors to moderate the tone of such comparisons throughout.

For example, the comparison of first passage times between simulation and experiment at L.342 (and similar instances

elsewhere) should be reframed: rather than claiming quantitative agreement, it is more appropriate to note that the model reproduces the correct order of magnitude or qualitative trend. This applies equally to the use of parameter values: where these are borrowed from three-dimensional models, the authors should acknowledge explicitly that the same numerical values may not hold in two dimensions.

1.5 Cite and engage with Minc et al. (Cell, 2011)

In the context of modeling spindle orientation in cells with complex geometries, the authors should cite and discuss Minc et al. (Cell, 2011), which demonstrated that a 2D cytoplasmic pulling force model can reproduce spindle orientation across a wide range of experimentally imposed cell shapes (including Figures 4 and 6A of that paper) as well as in cells with naturally complex geometries (Figure 6B). This work is directly relevant to the present manuscript and should be engaged with appropriately in both the Introduction and Discussion. Whether or not the authors choose to implement the Minc et al. model for the zebrafish cell data (see Comment 2.2 below) they should at minimum discuss this paper in the context of their own approach.

Comment 2: The treatment of cytoplasmic pulling forces requires further attention

2.1 Supplemental Figure S6 and its implications for the main conclusions

I appreciate the addition of Supplemental Figure S6, which presents simulations incorporating cytoplasmic pulling forces. Based on my reading of this figure, cytoplasmic pulling appears to perform comparably to MT pushing in reproducing the observed spindle behavior in zebrafish endothelial cells.

The fact that cytoplasmic pulling performs comparably to MT pushing in the simulations should be discussed transparently in both the Results and Discussion. The authors might, for example, frame this along the following lines: "although cytoplasmic pulling forces produce results comparable to MT pushing in our simulations, we consider cytoplasmic pulling to be less supported by current experimental evidence in this system, and therefore propose that the combination of MT pushing and junctional cortical pulling accounts for the observed spindle dynamics in zebrafish endothelial tip cells."

2.2 Correction regarding my previous comment #2

In their response, the authors interpret my citation of Kimura & Onami 2007 as a typographical error and assume I intended the 2005 paper. This is not correct. The reference I intended was Kimura & Onami 2007 (Journal of Cell Biology); the journal name was omitted in my previous comment only because the same paper had been cited in the immediately preceding point. The 2007 paper is directly relevant to the present work: it demonstrates that in *C. elegans*, RNAi depletion of cortical force generators not only fails to abolish centrations but actually accelerates it, and that spindle rotation still occurs under these conditions. The authors show that both position (i.e. centration) and orientation (i.e. rotation)-in wild-type and cortical force generator-depleted embryos-can be explained by a three-dimensional model combining cytoplasmic and cortical pulling forces. This paper therefore provides evidence that cortical pulling is not required for centration and may in fact oppose it, and that shape-dependent spindle rotation can occur independently of cortical pulling. In addition, the paper also constitutes an important precedent for the present manuscript, in that it demonstrates how the combination of two distinct force-generating mechanisms can account for both spindle position and orientation within a single modeling framework. These findings are directly relevant to the interpretation of the present manuscript and should be cited and discussed appropriately.

Comment 3: Specific comments on Figures 5 and 6

3.1 Figure 5C and 5D: clarify the time axis

The legend for Figures 5C and 5D should specify what time = 0 corresponds to (e.g., nuclear envelope breakdown, onset of prometaphase) and what event defines the end of the measurement period. Units should also be indicated on the time axis.

3.2 Parameter dependence in Figure 6 simulations

The dependence of the Figure 6 simulation results on key model parameters should be discussed more explicitly in the main text. In particular:

(3.2.1) It is important to clarify whether, and where, the cortex is saturated with respect to force generator occupancy by astral MTs. Given the elongated geometry of these cells, MT reach will vary substantially along the cell axis: the central cortical region is likely to be well-sampled by MTs from both poles, and may be near saturation, while the distal tip regions are likely undersampled. The authors should discuss how this spatial variation in MT-cortex contact probability affects the force balance and the simulation outcomes.

(3.2.2) Regarding MT length distributions (a point I also raised in my first-round review): if the dynamic instability parameters are held constant across conditions, the steady-state MT length distribution will be determined by those parameters regardless of the initial distribution assumed. The authors should show the steady-state length distribution for the conditions used in the Figure 6 simulations, so that readers can assess what MT lengths are actually being sampled.

3.3 Comparison of simulation and experiment: include trajectory-level analysis

The current comparison between simulation predictions and experimental observations is based on final spindle angle and position. While this is informative, the dynamics leading to the final state are also relevant and should be compared. For spindle position, the key question is whether the spindle moves in the correct direction (toward the proximal/junction end) from its initial

position. For spindle angle, similarly, the question is whether the angle decreases from its initial angle. I would ask the authors to quantify and compare the directional trends in spindle displacement and angle change between simulation and experiment, in addition to the final values.

1. Please note that it is The EMBO Journal policy for the transcript of the editorial process (containing referee reports and your response letters) to be published as an online supplement to each paper. If you should prefer removal of any referee-only figures included in the point-by-point response(s), e.g. because they may still be used for future publication or because they have been reproduced from published work by others, please do let us know immediately via response email. More information is available here: https://www.embopress.org/transparent-process#Review_Process

We have no concerns about this.

2. In the "Data Availability" section, please state: "This study includes no data deposited in external repositories". Further information on the format can be found at <https://link.springer.com/partners/embo-press/editorial-policies#Data%20availability%20statement>.

We've added the sentence as requested.

3. Please ensure that the figures are cited in sequential order. Currently, Fig 2B-C is called out before Fig 4 in the manuscript text.

The out of sequence calls have been corrected.

4. Please correct the nomenclature of the expanded view figures to "Figure EV1" and "Figure EV2".

This has been corrected.

5. For the Appendix items, please correct the nomenclature to "Appendix Table S1" and "Appendix Figure S1" etc. Please remove movie legends from the Appendix file.

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Ieva

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Referee #3:

The two principal concerns I raised in the first round-the overclaiming of a "generalizable" model and the exclusion of cytoplasmic pulling forces-have been addressed in a manner I find broadly satisfactory. Withdrawing the generalizability claim and repositioning the model as a tool suited to the analysis of complex cell geometries such as those of vascular endothelial cells is an appropriate response, and I accept this reframing. The addition of Supplemental Figure S6 exploring cytoplasmic pulling is also appreciated.

That said, the manuscript still carries significant structural weight from its original framing, and I believe a further round of revision is needed before it can be accepted.

We are grateful to this Reviewer for their time and effort in carefully reviewing our manuscript. We the authors have carefully considered their comments and provide point-by-point responses below.

It is our opinion that these requests do not generally identify errors or deficiencies in the revised work. Instead, the reviewer appears to seek authorship-level control over the manuscript: they ask us to reorganize the paper around their preferred framing, subordinate substantial parts of the study that remain central to our scientific argument, and reshape the conclusions according to the paper as they would have written it. Several comments overlook analyses added in direct response to the first review. In one case a new rationale is assigned to an earlier criticism and we are faulted for not having addressed it adequately. While we have taken the comments seriously, we do not think that the reviewer can be fully satisfied.

Comment 1: The manuscript's focus and structure should be realigned with the revised framing. Now that the authors have repositioned this as a study of spindle orientation and positioning in complex cell geometries-with the zebrafish vascular endothelial cell as the central application-the structure of the manuscript should reflect this. At present, the paper devotes considerable space (currently spanning approximately four pages of the Results) to simulations of *Drosophila* and *C.*

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We have changed it to 'A 2D computational model for spindle orientation and positioning applicable to diverse cell geometries' as suggested.

1.2 Substantially condense the treatment of Figures 2 and 3

The simulations of *Drosophila* follicular epithelial cells, neuroblasts, and the *C. elegans* embryo serve a legitimate purpose as validation of the model against well-established experimental observations. However, given the revised focus of the paper, this material should be condensed substantially so that the manuscript reaches the zebrafish data more efficiently. I would suggest treating these results as model validation and reducing their coverage in the main text accordingly. Moving Figures 2 and 3 to the Supplemental section is one option the authors may wish to consider, though I do not require this; what I do require is that the current level of detail in the main text be significantly reduced.

We do not agree with the reviewer. Figure 2 identifies threshold behavior that we test experimentally, while Figure 3 evaluates rotation, centration, asymmetric displacement, and the response to spindle severing. These figures therefore form part of the mechanistic argument and explore conserved roles for microtubule pushing forces across cell types. They do not simply serve as technical validation.

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Figure 4 presents a detailed mechanistic analysis of how MT pushing prevents spindle stalling and promotes dynamicity in follicle cells. In the current structure, this analysis precedes the zebrafish data (Figures 5 and 6) that motivates it. I would suggest that Figure 4 could be positioned after Figures 5 and 6, so that the mechanistic dissection follows naturally from the observation in Figure 6 that pushing forces are needed to prevent over-correction by junctional pulling. This reordering would strengthen the logical flow of the paper. I offer this as a suggestion rather than a requirement, as the authors are in the best position to judge the overall narrative.

We understand and appreciate the suggestion. We would prefer to keep Figure 4 in its current position.

1.4 Tone down quantitative comparisons between simulation and experiment

Because the model is two-dimensional and the parameter values used were originally derived mostly in the context of three-dimensional models, precise quantitative agreement between

simulation outputs and experimental measurements cannot be meaningfully claimed. I would ask the authors to moderate the tone of such comparisons throughout.

For example, the comparison of first passage times between simulation and experiment at L.342 (and similar instances elsewhere) should be reframed: rather than claiming quantitative agreement, it is more appropriate to note that the model reproduces the correct order of magnitude or qualitative trend. This applies equally to the use of parameter values: where these are borrowed from three-dimensional models, the authors should acknowledge explicitly that the same numerical values may not hold in two dimensions.

Reviewer 3 previously requested a quantitative comparison of model timescales with experimental data, commenting: 'This discrepancy deserves more attention, are the timescales qualitatively or quantitatively different? A brief quantitative comparison would clarify whether this is a minor calibration issue or a more substantive model limitation.' We performed this analysis and reported that parameter combinations within the physiologically plausible range produce FPTs on the order of 300 seconds, consistent with the ~5 minute orientation timescale reported in Peyre et al. (2011). The current comment now requests that we moderate the tone of this same comparison on the grounds that precise quantitative agreement cannot be meaningfully claimed from a 2D model. We respectfully note that we had initially framed this comparison in qualitative terms precisely because of the 2D limitation, and that the quantitative framing was introduced in direct response to the previous reviewer request.

1.5 Cite and engage with Minc et al. (Cell, 2011)

In the context of modeling spindle orientation in cells with complex geometries, the authors should cite and discuss Minc et al. (Cell, 2011), which demonstrated that a 2D cytoplasmic pulling force model can reproduce spindle orientation across a wide range of experimentally imposed cell shapes (including Figures 4 and 6A of that paper) as well as in cells with naturally complex geometries (Figure 6B). This work is directly relevant to the present manuscript and should be engaged with appropriately in both the Introduction and Discussion. Whether or not the authors choose to implement the Minc et al. model for the zebrafish cell data (see Comment 2.2 below) they should at minimum discuss this paper in the context of their own approach.

We are baffled by the reviewer's comment, particularly "(w)hether or not the authors choose to implement the Minc *et al.* model for the zebrafish cell data." We cited and discussed Minc *et al.* (2011) in the revised Results. In direct response to the reviewer's original Comment #2, we implemented length-dependent cytoplasmic pulling (the Minc *et al.* model) in the experimentally measured zebrafish endothelial-cell geometries. These simulations are reported in Supplemental Figure 6 and demonstrate that cytoplasmic pulling can reproduce geometry-dependent spindle orientation in these cells.

Comment 2: The treatment of cytoplasmic pulling forces requires further attention

2.1 Supplemental Figure S6 and its implications for the main conclusions

I appreciate the addition of Supplemental Figure S6, which presents simulations incorporating cytoplasmic pulling forces. Based on my reading of this figure, cytoplasmic pulling appears to perform comparably to MT pushing in reproducing the observed spindle behavior in zebrafish endothelial cells.

The fact that cytoplasmic pulling performs comparably to MT pushing in the simulations should be discussed transparently in both the Results and Discussion. The authors might, for example, frame this along the following lines: "although cytoplasmic pulling forces produce results comparable to MT pushing in our simulations, we consider cytoplasmic pulling to be less supported by current experimental evidence in this system, and therefore propose that the combination of MT pushing and junctional cortical pulling accounts for the observed spindle dynamics in zebrafish endothelial tip cells."

We are puzzled by this comment. The following paragraph appears in the revised manuscript already:

Our results suggest that spindle orientation in angiogenic endothelial cells is dictated primarily by cell geometry. If so, an independent mechanism that encodes cell geometry should produce a similar outcome. While extensive experimental evidence supports microtubule pushing (Dogterom & Yurke, 1997; Holy et al, 1997; Tran et al, 2001; Janson et al, 2003; Daga et al, 2006; Laan et al, 2012; Sulerud et al, 2020; Cueff et al, 2024), length-dependent cytoplasm-based microtubule pulling has also been proposed to translate cell shape into spindle orientation (Kimura & Onami, 2005; Minc et al, 2011). We therefore asked whether this independent mechanism could orient the spindle in irregularly shaped angiogenic endothelial cells. We implemented cytoplasmic pulling forces following the length-dependent formulation $F_{cyto} = k \times L$, where k is a scaling coefficient. All microtubules are subject to cytoplasmic pulling and pushing is excluded. We tested three values for k : 0.05, 1, and 10 pN/ μ m. The first two are consistent with a range of values used previously and considered biologically relevant (Kimura & Onami, 2005). Spindle orientation along the cell long axis was achieved at all three values, though it was marginally less accurate at 10 pN/ μ m (Supplemental Figure 6). These results support the idea that spindle orientation in angiogenic endothelial cells can be dictated by cell geometry without the need for force generators pulling from the cortex. Importantly, we do not consider these results to be evidence for cytoplasmic pulling in our system. Angiogenic endothelial cells are not large, and their width ($\sim 10 \mu$ m) is comparable to the length of the spindle. Astral microtubules are therefore expected to contact the cortex frequently, and we therefore expect cortical interactions to predominate spindle orientation and positioning in these cells.

We don't know how much more transparent that needs to be. We also address cytoplasmic pulling directly in the Introduction of the revised manuscript:

Dynein-dependent pulling of astral MTs has also been proposed to arise within the cytoplasm rather than at the cortex. This is suggested by observations in large embryonic cells, including Xenopus, zebrafish, and sea urchin embryos, in which asters can begin to move before astral MTs contact the cortex (Wühr et al. 2010; Tanimoto et al. 2016, 2018). However, there is less support for the possibility that cytoplasmic forces contribute to spindle positioning and orientation in smaller cells, in which astral microtubules are expected to contact the cortex regularly. Although cytoplasmic pulling has been suggested to explain centrosome migration in the one-cell C. elegans embryo (A. Kimura and Onami 2005), subsequent work found no evidence for such a mechanism (Wu et al. 2024). The mechanistic basis for cytoplasmic pulling in this cell also remains unclear. One proposal is that dynein associated with intracellular organelles generates length-dependent pulling forces on astral MTs (K. Kimura and Kimura 2011), but large-scale

imaging in Xenopus egg extracts showed that MTs and cytoplasmic organelles largely co-migrate rather than moving past one another (Pelletier et al. 2020).

2.2 Correction regarding my previous comment #2

In their response, the authors interpret my citation of Kimura & Onami 2007 as a typographical error and assume I intended the 2005 paper. This is not correct. The reference I intended was Kimura & Onami 2007 (Journal of Cell Biology); the journal name was omitted in my previous comment only because the same paper had been cited in the immediately preceding point. The 2007 paper is directly relevant to the present work: it demonstrates that in *C. elegans*, RNAi depletion of cortical force generators not only fails to abolish centration but actually accelerates it, and that spindle rotation still occurs under these conditions. The authors show that both position (i.e. centration) and orientation (i.e. rotation)-in wild-type and cortical force generator-depleted embryos-can be explained by a three-dimensional model combining cytoplasmic and cortical pulling forces. This paper therefore provides evidence that cortical pulling is not required for centration and may in fact oppose it, and that shape-dependent spindle rotation can occur independently of cortical pulling. In addition, the paper also constitutes an important precedent for the present manuscript, in that it demonstrates how the combination of two distinct force-generating mechanisms can account for both spindle position and orientation within a single modeling framework. These findings are directly relevant to the interpretation of the present manuscript and should be cited and discussed appropriately.

This is a misrepresentation. The Reviewer's original comment was: "It is well established that cytoplasmic pulling forces can encode cell shape information and direct spindle orientation (e.g., Minc et al. Cell 144, 414-426, 2011; Kimura & Onami 2007)" It is the 2005 paper "Computer simulations and image processing reveal length-dependent pulling force as the primary mechanism for *C. elegans* male pronuclear migration" that establishes cytoplasmic pulling forces as encoding shape information. They now use a *different justification* to claim that the 2007 paper "Local cortical pulling-force repression switches centrosomal centration and posterior displacement in *C. elegans*" was the intended reference.

We cannot score if the goalposts are moving.

Comment 3: Specific comments on Figures 5 and 6

3.1 Figure 5C and 5D: clarify the time axis

The legend for Figures 5C and 5D should specify what time = 0 corresponds to (e.g., nuclear envelope breakdown, onset of prometaphase) and what event defines the end of the measurement period. Units should also be indicated on the time axis.

We thank the reviewer for this comment. We have updated the legends for Figures 5C and 5D to specify that $t = 0$ corresponds to the time point at which the mitotic spindle is fully assembled, and that the measurement period ends at anaphase onset. We also altered the time stamps on figure 5 A' accordingly and added "Selected stills (every 84 seconds) from a movie, 0 seconds represents the first frame with a complete mitotic spindle and corresponds to the 0 time point in (C) and (D)." to the figure legend.

3.2 Parameter dependence in Figure 6 simulations

The dependence of the Figure 6 simulation results on key model parameters should be discussed more explicitly in the main text. In particular:

(3.2.1) It is important to clarify whether, and where, the cortex is saturated with respect to force generator occupancy by astral MTs. Given the elongated geometry of these cells, MT reach will vary substantially along the cell axis: the central cortical region is likely to be well-sampled by MTs from both poles, and may be near saturation, while the distal tip regions are likely undersampled. The authors should discuss how this spatial variation in MT-cortex contact probability affects the force balance and the simulation outcomes.

We would offer one clarification about the model geometry. Astral MTs do not nucleate isotropically in our model: they emanate from each pole within a restricted cone of angle β (see Appendix Table S1), on the assumption that MTs directed towards the metaphase plate are obstructed by the chromosome mass. Cortical sampling is therefore set jointly by this restriction and by pole-to-cortex distance, and the equatorial cortex lies at the edge of the nucleation cone from either pole rather than being preferentially sampled from both. Contact frequency is also not the same quantity as delivered force, since pushing scales as $F = \min(\pi^2\kappa/L^2, F_{\max})$. We emphasise that this spatial variation is the mechanism rather than a confound: the geometric readout exists because MTs contacting nearby cortex push harder than those contacting distant cortex. We have added a short passage to the Results making this explicit: *“This readout arises naturally from the inverse-square dependence of pushing force on MT length: MTs growing toward nearby cortex are shorter and push harder, while those reaching distant cortex are longer and push less, so the force each cortical region receives is set by its distance from the spindle pole.”* We have not attempted a direct measurement of local force-generator occupancy, but note that the conditions compared in Figure 6 use identical FG density and differ only in spatial arrangement, and that predicted angle and position are invariant across the $N_{\text{MT}}-N_{\text{FG}}$ pairs in Expanded View Figure 2E,F. We hope that this satisfactorily addresses the reviewer's concern.

(3.2.2) Regarding MT length distributions (a point I also raised in my first-round review): if the dynamic instability parameters are held constant across conditions, the steady-state MT length distribution will be determined by those parameters regardless of the initial distribution assumed. The authors should show the steady-state length distribution for the conditions used in the Figure 6 simulations, so that readers can assess what MT lengths are actually being sampled.

The reviewer is correct and we have revised the Methods to state this explicitly: *“With dynamic instability parameters held constant, the initial MT length distribution is a transient that relaxes toward a steady state set by the dynamic instability rates. This consideration is particularly relevant in zebrafish ISV cells, where cell shape changes dynamically during the simulation — astral MTs are censored at the pole-to-cortex distance, which itself varies over time, so the lengths actually sampled depend jointly on dynamic instability rates and the evolving cell geometry and it is this geometric dependence of the sampled lengths that converts cell shape into a net torque.”*

We would add, however, that the free-solution steady state is not the distribution that matters mechanically here: an astral MT cannot extend beyond the pole-to-cortex distance along its direction of growth, so the distribution actually sampled is censored at that distance, and in cells of these dimensions (width $\sim 10\ \mu\text{m}$, comparable to spindle length) many MTs meet the cortex well before reaching the lengths they would attain unconfined. The sampled lengths are therefore

set jointly by the dynamic instability rates and by cell shape, and differ between cortical regions within the same cell. Since pushing force falls as $1/L^2$, it is this geometric dependence of the sampled lengths that converts cell shape into a net torque; it is also why model outputs are insensitive to the assumed initial distribution (Supplemental Figure 7A), the sole exception being the 1 μm constant-length condition, in which orientation is delayed because MTs must first grow far enough to reach the cortex.

3.3 Comparison of simulation and experiment: include trajectory-level analysis
The current comparison between simulation predictions and experimental observations is based on final spindle angle and position. While this is informative, the dynamics leading to the final state are also relevant and should be compared. For spindle position, the key question is whether the spindle moves in the correct direction (toward the proximal/junction end) from its initial position. For spindle angle, similarly, the question is whether the angle decreases from its initial angle. I would ask the authors to quantify and compare the directional trends in spindle displacement and angle change between simulation and experiment, in addition to the final values.

We appreciate the reviewer's point and agree that direction of travel is a meaningful test. It is, however, already embedded in the comparisons presented: each of the twelve geometries is simulated from its own experimentally observed initial spindle angle and position, so the comparisons in Figure 6B,C are between matched initial conditions, and agreement in final angle and position is equivalent to agreement in net displacement and net angular change from a common starting point. Trajectory-level data are also already shown: observed angle and position time series for all twelve cells individually (Figure 5C,D); full per-cell simulated distributions under each condition (Supplemental Figure 5); and individual simulation trajectories for the *C. elegans* validation cases (Supplemental Figure 3). A formal statistical treatment of the time-resolved dynamics raises questions about the temporal structure of the force balance that go beyond the orientation and positioning claims made here, and we politely suggest that it would be better pursued as a dedicated analysis.

Dear Dan and Holly,

Thank you for submitting the final version of your manuscript and for incorporating your responses to the editorial and reviewers' requests. From my side, I find that the remaining issues have been clarified sufficiently. I am now pleased to inform you that your manuscript has been accepted for publication. Congratulations on a nice study!

Before we forward your manuscript to our publishers, there are a couple of aspects that I still need your input on:

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2. The synopsis image that you kindly provided becomes rather fuzzy when resized to the required dimension of 550x300 pixels (please see in the attachment). I am afraid that I also noticed a typo: on the right-hand side, it should say "junctionally". It would be great if you could send me an updated image with these issues corrected.

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Once I have received your input on the final points, I will be happy to forward your manuscript to our publishers for typesetting.

Thank you for this interesting contribution to The EMBO Journal!

Best wishes,

Ieva

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- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
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