Diverse mechanisms regulate contractile ring assembly for cytokinesis in the two-cell *Caenorhabditis elegans* embryo

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MS TITLE: The Ran pathway uniquely regulates cytokinesis in cells with different fates in the early C. elegans embryo

AUTHORS: Imge Ozugergin, Karina Mastronardi, Christopher Law, and Alisa J Piekny
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

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

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

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

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

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to

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all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

Cell divisions occurs in wide variety of cell shapes, tissue context and developmental cell fates. While the process of cytokinesis has been extensively studied in single cells, zygotes or cell monolayers, the role of cell fate and developmental context in the regulation of cytokinesis is only beginning to be understood. Generally local activation of the small GTPase RhoA at the cell cortex midway between the two spindle poles promotes assembly of the actomyosin cytokinetic ring that drives the separation of the two daughter cells. Additionally, contractile dynamics in the polar cortex and cortical flows as well chromatin derived cues have been reported to contribute to cytokinetic ring assembly. The latter signals appear to depend on cellular Ran-GTP gradients and interaction of the cytoskeletal cross-linker protein Anillin with importin at the cell cortex. How this latter interaction contributes to cytokinetic ring dynamics is still poorly understood.
In this manuscript the authors report differences in cytokinetic ring kinetics in the two blastomeres of the two-cell stage C. elegans embryo, where each blastomere exhibits a distinct cell fate. They investigate the impact of perturbing cell fate, cell size, and the Ran-GEF Rcc-1 and selected importins on these differential kinetics in an effort to explain the mechanistic differences that contribute to the differences in cytokinetic ring kinetics in these differentially fated cells By evaluating myosin levels at the site of furrow ingression the authors propose that the difference in ring dynamics is due to differences in local myosin levels and organization and that that the blastomeres exhibit different sensitivity to regulation by the Ran pathway.
Most of the presented findings are novel and interesting, and potentially suitable for publication in JCS. The experimental execution is thorough and the quantification of the results is largely solid but could benefit from some improvements or additions as detailed below. My main concern is that, perhaps in an effort to provide a simple mechanistic explanation for their observations, the authors propose a likely oversimplified model that in my opinion is insufficiently supported, and at times contradicted, by their own data. Specifically, the authors propose the existence of various thresholds of myosin levels that can lead to different kinetics of ring assembly that explain the observed differences closure kinetics in various analyzed conditions. Yet for example RAN-3 depleted P1 cells exhibit similar or slightly lower myosin levels than control P1 cells yet ring assembly is faster. Similarly, PAR-1 depleted cells have lower myosin levels than AB cells yet significantly faster initiation kinetics. Both observations contradict their summary model. To support this model the authors should provide a more direct examination of the effects of myosin levels on initiation kinetics (see detailed suggestions below) or at least highlight that their model fails to explain a significant fraction of their observations. In general, I support the publication of this manuscript in JCS if the following points are addressed.

Comments for the author

Major points:
1) In accordance with previous work the authors describe 3 distinct stages of ring closure, and the kinetics of all three appear to differ between AB and P1 and in the various perturbations throughout the manuscript, yet only the quantifications for the ring assembly phase is consistently shown. Quantification for the other phases should be provided, and the observed difference in kinetics should be addressed in their interpretation of the difference in ring kinetics among conditions. Also, a more detailed description of how the 3 phases were defined experimentally needs to be provided in the Results section.
2) The authors propose that the observed differences in ring assembly kinetics can be explained by a model in which peak myosin levels explain assembly kinetics. This is based on correlative evidence, and a substantial proportion of the data seem to contradict this model. A direct test of this model would be to change myosin levels experimentally in control cells and investigate the effects on ring kinetics. Partial depletion of NMY-2 could be used to decrease myosin levels and/or worm strains with endogenously tagged myosin could be compared to strains in which tagged myosin is over-expressed from an exogenous locus. Excess myosin should also be seen at the cytokinetic ring following depletion of myosin phosphatase.

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3) Instead of / in addition to reporting fluorescence intensity for a single timepoint as a function of position along the cortex the authors should quantify the area under the curve per time point and report total fluorescence intensity in the furrow as a function of time which seems a more relevant metric when examining the role of myosin levels in explaining differences in cytokinetic ring kinetics.

Minor comments (in order as they appear in the manuscript)
1) It is not clear to me why ring asymmetry was differentially scored in x and y and what timepoint during ring ingression was chosen and why for the quantification in Figure S1C. I would recommend to instead use the distance of the cytokinetic ring center from the cell center adjusted for cell width as a quantitative measurement of ring asymmetry.
2) The correlation, or in most cases lack thereof, between cell size and fluorescence intensity within a similarly fated blastomeres within the same condition does not appear to help explain the difference in ring kinetics and can therefore be omitted or at least moved to a supplemental figure.
3) It is not clear if failed cytokinesis in ect-2 RNAi embryos refers to embryos that fail to initiate ingression or embryos that initiate but fail completion of cytokinesis. This needs to be clarified. Also a quantitative comparison between cytokinesis competent and failing embryos regarding myosin organization and kinetics (if “failing” embryos attempt furrowing) would be helpful to interpret the results since myosin levels appear similar in ECT-2 depleted cells that are furrow-competent and depleted AB cells failing cytokinesis.
4) The absence of flows in both AB and P1 blastomeres in partial ECT-2 depletions, in conjunction with asymmetric ring closure in depleted AB cells, suggests that flows do not drive ring asymmetry contrary what the authors stated earlier.
5) When assessing the effect of polarity on ring kinetics, the authors show that PAR-1 and PAR-3 depleted cells have different kinetics, yet they only present intensity and myosin organization data for PAR-1 depletions. Parallel presentation of data for PAR-3 depletion would be helpful to understand the differences between these conditions.
6) The sentence: “The rings in tetraploid P1 cells closed symmetrically, but they were more asymmetric than in diploid cells (Figure 4G; Figure S1C)”, is confusing.
7) To assert “… this NLS is conserved in ANI-1, and we propose that binding to importin-ß similarly facilitates its cortical recruitment and function during cytokinesis (Figure 6B),” the authors need to not only show importin-anillin binding but also show that depletion of importin affects anillin localization to the furrow in individual blastomeres.
8) For RAN-3 depletions, the authors state “The levels of myosin were similar to control cells, but distributed over a broader area, suggesting a change in its organization that facilitates contractility.” It is not immediately evident from the data presented that the accumulation in AB cells is significantly wider. Also the peak levels in P1 are significantly below the threshold the authors claim is the threshold necessary to support ingression yet the furrow ingresses in these cells. This seems an interesting observation that should be addressed.
9) When the authors state ”However, data from a number of labs suggest that importin-α and -ß can bind independently to NLS-containing proteins (Ozugergin & Piekny, 2020)” they need to cite work from some of these other labs.
10) The differential rescue of the RCC-1 depletion phenotype by ANI-1 RNAi in P1 vs AB blastomeres is very interesting but not fully explored by the authors. They should at least speculate about the reason for this difference. Perhaps comparing anillin localization in P1 and AB in the presence or absence of RCC-1, or myosin levels and organization in the rescue experiment, would provide valuable insights into the differences between AB and P1 ring kinetics.
11) It is noteworthy that many of the perturbations besides tetraploidy cause changes in blastomere size. For example, PAR-1 depleted embryos are significantly smaller than PAR-3 RNAi embryos yet ingression kinetics are similar. RCC-1 embryos seem to have equally sized blastomeres yet likely normal polarity and similar closure kinetics. How do the authors reconcile these observations with their other results when modulating cell size?

Reviewer 2

Advance summary and potential significance to field

The authors investigate how Ran regulates cytokinesis in early worm embryo divisions. The manuscript comes across as somewhat unfocused, exploring the roles of cell fate, cell size and the Ran pathway on cytokinesis in the 2 cell divisions. While differences in AB and P1 divisions may

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have some purpose, I missed any insights generated from this study. In general, the report is fairly descriptive, carefully measuring aspects of cytokinesis and observing changes in several somewhat unrelated conditions. While the subject is of interest, there lacks a clear mechanistic insight outside some subtle differences in the effects of depleting different Ran regulators for reasons that are not well explained. I have several concerns:

Comments for the author

Major Concerns:
1. The title indicates a “unique regulation” by Ran, but this is vague. What is regulated? What specifically is Ran doing? There is little analysis of Ran components other than RNAi depletion of different players of the pathway. The one potential target, ANI-1, is not characterized much further than a genetic interaction experiment.
2. The “ring assembly onset” phase is not well defined, how can this be measured using only a membrane and DNA marker as in figure 1? Is this just the time between anaphase onset and invagination of the furrow? It seems that directly observing ring components would be important to measure the formation of the ring.
3. When measuring ring ingression symmetry (Fig1G etc.), it does not appear to take into consideration that the border between cells might move during furrow ingression such that the original ring position is not an accurate indicator of how far the furrow ingresses from the cell boundary.
4. Line 345: the prediction that differences in myosin is due to RhoA is stated.
Are there also differences in myosin protein levels? This might be important for understanding the PAR experiments also. If there are different myosin protein levels, then disrupting PAR would equalize myosin levels and impact cytokinesis.
5. It might be more precise to use a temperature sensitive ect-2 mutant rather than an unspecified reduction in ect-2 by RNAi to more accurately characterize its activity during these divisions.
6. The experiments addressing the role of cell size seem to imply that changes to ploidy will also affect the Ran pathway. Therefore, it is not clear if these experiments indicate something about spatial dimensions of the cell, spindle and cortex, or an alteration in the Ran gradient. This is never addressed in the manuscript.
7. In figure S5A, images are shown that polarity is normal. However, it is also clear that the normal positions of daughter cells relative to each other at the 4 cell stage are significantly impacted. In Figure 5E, the P1 furrow is significantly mispositioned, is this due to changes in contacts between the cells?
Given that Ran regulates the spindle, and the anaphase spindle elongation in AB drives the relative positions of daughter cells at the 4-cell stage, it concerns me that perhaps the spindle is abnormal in these cells. This also brings up the possibility that Ran affects the spindle, which more indirectly affects cytokinesis. Why is cell shape/overall positioning affected?
8. It seems important to show that the Ran system affects anillin localization instead of only showing genetic interaction data.
9. Several Ran players are depleted by RNAi, but the Ran gradient and/or localization and function of any individual Ran component is not directly studied in the manuscript. It would seem to be important to characterize how these players work in a direct way to understand how downstream targets might be affected.

Minor concern:
10. In the intro, lines 95-98, Ran is proposed to enhance anillin recruitment to the cortex. However, when Ran regulates spindle assembly, it releases spindle factors from importin binding near the chromatin if I understand correctly. How can this pathway enhance anillin recruitment to the cortex if importins will be released from binding near chromatin?
11. The finding that cell fate, size and Ran involvement has been previously described in other studies. It’s not immediately clear how these results change or improve on what was previously known from the introduction.
12. What has already been investigated regarding differences in cytokinesis regulation in different cell types in the embryo is not well described and compared with the new insights from this study.
13. It is difficult to see the “myosin and actin levels were higher along the anterior cortex compared to the posterior” in Fig1E, perhaps arrows to highlight this? It is also hard to compare this with Fig 2E to see the difference.

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14. Line 349: How long was this RNAi depletion done, what was the percent cytokinesis failure under these conditions?
15. Line 457: I did not see the Ran experiment in figure S4.
16. I do not understand what the finding that imb-1/ima-3 co-depletion means as it pertains to furrow ingression rate. Are they having opposite effects on the same targets, counteracting each other or something else?

First revision

Author response to reviewers' comments

Reviewer 1

Major points:
1)In accordance with previous work the authors describe 3 distinct stages of ring closure, and the kinetics of all three appear to differ between AB and P1 and in the various perturbations throughout the manuscript, yet only the quantifications for the ring assembly phase is consistently shown. Quantification for the other phases should be provided, and the observed difference in kinetics should be addressed in their interpretation of the difference in ring kinetics among conditions. Also, a more detailed description of how the 3 phases were defined experimentally needs to be provided in the Results section.

We appreciate the reviewer's suggestion. We added quantification of the other phases for control AB and P1 cells (Fig. 1C, S2D), and a description of how these were measured is provided in the methods under 'Quantitative data analysis' and referred to in the main text in relation to Fig. 1C. While there are differences between AB and P1 cells in all phases, we have focused on the ring assembly phase in this manuscript as this is when the Ran pathway is most likely able to regulate the organization of actomyosin [we also included a comparison of all phases for ran-3(RNAi) in Fig. S7]. The parameters controlling later phases are likely to be quite different and extend beyond this study. We also changed the title to reflect the emphasis on ring assembly.

2)The authors propose that the observed differences in ring assembly kinetics can be explained by a model in which peak myosin levels explain assembly kinetics. This is based on correlative evidence, and a substantial proportion of the data seem to contradict this model. A direct test of this model would be to change myosin levels experimentally in control cells and investigate the effects on ring kinetics. Partial depletion of NMY-2 could be used to decrease myosin levels and/or worm strains with endogenously tagged myosin could be compared to strains in which tagged myosin is over-expressed from an exogenous locus. Excess myosin should also be seen at the cytokinetic ring following depletion of myosin phosphatase.

We appreciate this excellent suggestion. During the revision process we have refined our understanding of how ring assembly might be differently controlled in AB vs. P1 cells. As recommended, we partially depleted NMY-2 and observed that even though ring closure was delayed as expected, the relative differences in ring assembly kinetics between AB and P1 cells were retained, similar to ECT-2 depletion (Figure S3C vs. 2B). This helped us to realize that the differences in myosin levels alone do not govern the rate of assembly, which also depends on other factors that could be RhoA-independent and impact myosin organization. The increase in myosin levels in the tetraploid P1 cells, which have kinetics more like control AB cells also revealed that these other factors may play more crucial rules in ring assembly when myosin levels are low vs. above threshold. Testing this model would require cell-specific tools which is beyond the scope of the revision, but would be interesting to do in a follow-up study.

3)Instead of / in addition to reporting fluorescence intensity for a single timepoint as a function of position along the cortex the authors should quantify the area under the curve per time point and report total fluorescence intensity in the furrow as a function of time which seems a more relevant metric when examining the role of myosin levels in explaining differences in cytokinetic ring kinetics.

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We thank the reviewer for this suggestion. We have added the' area under the curve' measurements to account for differences in breadth (Figure 2F). As the reviewer suggested, this indeed gives us a more accurate measurement of the changes in myosin levels vs. only reporting on peak intensity differences. When considering both values, this helps make sense of how myosin levels correlate with the observed phenotypes. For example, we see higher peaks and areas of levels of myosin in AB vs. P1 cells. These values decrease after ect-2 RNAi and are higher in the tetraploid embryos as expected. The peak values did not change in ran-3 (RNAi) AB and P1 cells, but we did see a mild increase in accumulation in AB cells, which has helped us to refine our understanding of how Ran-GTP could influence actomyosin organization vs. levels per se. As explained above, we did not do measurements over time because our emphasis is on the early phase of ring assembly when Ran is most likely to regulate cytokinesis.

Minor comments (in order as they appear in the manuscript):
1) It is not clear to me why ring asymmetry was differentially scored in x and y and what timepoint during ring ingression was chosen and why for the quantification in Figure S1C. I would recommend to instead use the distance of the cytokinetic ring center from the cell center adjusted for cell width as a quantitative measurement of ring asymmetry.

The timepoints have been clarified, and analysis done as suggested (see methods 'Quantitative data analysis'; Fig S3D).

2) The correlation, or in most cases lack thereof, between cell size and fluorescence intensity within a similarly fated blastomeres within the same condition does not appear to help explain the difference in ring kinetics and can therefore be omitted or at least moved to a supplemental figure.

The graphs for cells which lack correlation have been moved to Fig. S5.

3) It is not clear if failed cytokinesis in ect-2 RNAi embryos refers to embryos that fail to initiate ingression or embryos that initiate but fail completion of cytokinesis. This needs to be clarified. Also a quantitative comparison between cytokinesis competent and failing embryos regarding myosin organization and kinetics (if "failing" embryos attempt furrowing) would be helpful to interpret the results since myosin levels appear similar in ECT-2 depleted cells that are furrow-competent and depleted AB cells failing cytokinesis.

To clarify this for the reader, we have included the kinetics for ect-2(RNAi) AB and P1 cells that ingress, but then fail cytokinesis (Fig. S6A). We also included HILO imaging of failed ect-2(RNAi) AB and P1 cells (Fig. S6B), where we measured filament alignment to show that the organization is worse compared to those that succeed cytokinesis (Fig. S6E).

4) The absence of flows in both AB and P1 blastomeres in partial ECT-2 depletions, in conjunction with asymmetric ring closure in depleted AB cells, suggests that flows do not drive ring asymmetry contrary what the authors stated earlier.

We agree with the reviewer and have modified the text to acknowledge that multiple factors contribute to asymmetric ingression.

5) When assessing the effect of polarity on ring kinetics, the authors show that PAR-1 and PAR-3 depleted cells have different kinetics, yet they only present intensity and myosin organization data for PAR-1 depletions. Parallel presentation of data for PAR-3 depletion would be helpful to understand the differences between these conditions.

We also included measurements of myosin levels in PAR-3-depleted embryos (Fig. 3F), which were similar to PAR-1-depleted embryos.

6)The sentence: "The rings in tetraploid P1 cells closed symmetrically, but they were more asymmetric than in diploid cells (Figure 4G; Figure S1C)", is confusing.

The text has been edited accordingly.

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7)To assert "… this NLS is conserved in ANI-1, and we propose that binding to importin-ß similarly facilitates its cortical recruitment and function during cytokinesis (Figure 6B)," the authors need to not only show importin-anillin binding but also show that depletion of importin affects anillin localization to the furrow in individual blastomeres.

This is an excellent suggestion. To address this, we depleted embryos of IMB-1 and measured changes in the levels of ANI-1 (Fig. 6C). Indeed, in support of our model we see a decrease compared to control embryos. Since cells are particularly sensitive to loss of IMB-1, e.g. a sharp threshold of depletion causes chromosome segregation defects, we measured this in P0 vs. AB or P1 cells where we could see stronger differences.

8)For RAN-3 depletions, the authors state "The levels of myosin were similar to control cells, but distributed over a broader area, suggesting a change in its organization that facilitates contractility." It is not immediately evident from the data presented that the accumulation in AB cells is significantly wider. Also the peak levels in P1 are significantly below the threshold the authors claim is the threshold necessary to support ingression yet the furrow ingresses in these cells. This seems an interesting observation that should be addressed.

We have readjusted some of the measurement methods, and added area under the curve measurements to better describe the shape of the peak. When we consider both sets of measurements we see that there is no major difference in myosin in ran-3(RNAi) vs control P1 cells, while we see a minor increase in AB cells. This helped us to consider that the Ran pathway could impact ring assembly in different ways in AB vs. P1 cells. We do see an increase in filament organization in ran-3(RNAi) P1 cells via HILO imaging, supporting the faster kinetics. The differences in the Ran regulation of ring assembly in AB vs. P1 are supported by our other data for depletion of the importins and ANI-1.

9)When the authors state "However, data from a number of labs suggest that importin-α and -ß can bind independently to NLS-containing proteins (Ozugergin & Piekny, 2020)" they need to cite work from some of these other labs.

The original references have been added.

10)The differential rescue of the RCC-1 depletion phenotype by ANI-1 RNAi in P1 vs AB blastomeres is very interesting but not fully explored by the authors. They should at least speculate about the reason for this difference. Perhaps comparing anillin localization in P1 and AB in the presence or absence of RCC-1, or myosin levels and organization in the rescue experiment, would provide valuable insights into the differences between AB and P1 ring kinetics.

The reviewer raises an important point. We have added measurements of ANI-1 in AB vs. P1 cells in control embryos compared to those after RAN-3 depletion (Fig. 6E). Indeed, the difference in levels support our model that the pathway could regulate ANI-1 in AB cells, but not in P1 cells (or has a different threshold requirement). We have also strengthened our speculation of how the pathway may be regulating ring assembly in AB vs. P1 cells in the discussion.

11)It is noteworthy that many of the perturbations besides tetraploidy cause changes in blastomere size. For example, PAR-1 depleted embryos are significantly smaller than PAR-3 RNAi embryos yet ingression kinetics are similar. RCC-1 embryos seem to have equally sized blastomeres yet likely normal polarity and similar closure kinetics. How do the authors reconcile these observations with their other results when modulating cell size?

We think that both fate and size can influence ring assembly kinetics. While fate could lead to differences in myosin levels between cell types, and thresholds that more easily support assembly, size can further influence assembly through factors that could include the Ran pathway. The latter could play a stronger role when myosin levels are lower and closer to vs. well above thresholds, which occurs in the PAR-1 or PAR-3 depleted embryos. We have added a section in the discussion to describe models that could support our observations and the role of size.

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Reviewer 2

Major Concerns:

1. The title indicates a “unique regulation” by Ran, but this is vague. What is regulated? What specifically is Ran doing? There is little analysis of Ran components other than RNAi depletion of different players of the pathway. The one potential target, ANI-1, is not characterized much further than a genetic interaction experiment.

The reviewer raises a good point about using a more accurate title that better reflects the focus of the manuscript. Since the emphasis of the manuscript is on the regulation of ring assembly kinetics in AB vs. P1 cells, we changed the title to: ‘Diverse mechanisms regulate contractile ring assembly for cytokinesis in the two-cell C. elegans embryo’.

2. The “ring assembly onset” phase is not well defined, how can this be measured using only a membrane and DNA marker as in figure 1? Is this just the time between anaphase onset and invagination of the furrow? It seems that directly observing ring components would be important to measure the formation of the ring.

We have adjusted the description in the text to clarify the assembly phase and why we are able to define this phase using a membrane marker. We used the same phases described in Osorio et al., (2019), who used myosin as a cortical marker to characterize these phases. RhoA activity increases in the equatorial plane upon anaphase onset triggering an increase in the assembly of actomyosin filaments. Given that it takes time for this actomyosin to assemble into a ring, the assumption is that ‘assembly phase’ includes the majority of time after anaphase onset, and before a ring is sufficiently organized to generate force. To help the reader follow this logic, we have added a panel showing a kymograph of myosin localization in Fig. S2B. The changes in cortical myosin are visible to the eye during this phase in AB cells as filaments become more organized before initiation phase.

3. When measuring ring ingression symmetry (Fig1G etc.), it does not appear to take into consideration that the border between cells might move during furrow ingression such that the original ring position is not an accurate indicator of how far the furrow ingresses from the cell boundary.

We understand the reviewer’s concern. To address this, all of the P1 cells that lack shared AB cell boundaries were analyzed separately as ‘influence-free’ cells. As shown in Fig. S4, there was no difference in asymmetric ingression with these cells vs. the average of the total number of cells which included those with shared boundaries. We did not do this for ran-3(RNAi) cells, because all of the P1 daughters are influence-free.

4. Line 345: the prediction that differences in myosin is due to RhoA is stated. Are there also differences in myosin protein levels? This might be important for understanding the PAR experiments also. If there are different myosin protein levels, then disrupting PAR would equalize myosin levels and impact cytokinesis.

Indeed, the implication is that in cells with different fates this could lead to differences in myosin levels, which is equalized when fate is disrupted. This is one of the parameters contributing to differences in assembly kinetics between cells. This has been clarified this in the text.

5. It might be more precise to use a temperature sensitive ect-2 mutant rather than an unspecified reduction in ect-2 by RNAi to more accurately characterize its activity during these divisions.

Theoretically a ts allele of ect-2 would be useful, but it is not clear if the ts allele of ect-2 is fast-acting enough (e.g. seconds) for our needs. Studying ect-2 function in the early embryo is difficult, because it is maternally required in the germline and P0 zygote. Since AB and P1 cells divide within minutes, it would be challenging to precisely upshift and knock down ect-2 function sufficiently to disrupt division in only the AB and P1 cells. This is the same challenge we currently have with RNAi. We would have a different set of parameters that would require extensive troubleshooting, and likely would not alter the outcome compared to titrating RNAi.

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6. The experiments addressing the role of cell size seem to imply that changes to ploidy will also affect the Ran pathway. Therefore, it is not clear if these experiments indicate something about spatial dimensions of the cell, spindle and cortex, or an alteration in the Ran gradient. This is never addressed in the manuscript.

We have changed the wording regarding the tetraploid experiments and clarified the relationship between different parameters. The increase in ploidy led to an increase in myosin levels, and likely other proteins specific to AB and P1 cells given that they retain their fates. This finding helped to reveal that sufficient levels of myosin can override the delayed assembly typically seen in control P1 cells. However, we still observed a negative correlation between duration of ring assembly and cell size in these cells, suggesting that the mechanisms responding to size are preserved. We propose that this could occur through the Ran pathway, where the Ran gradient can change in response to ploidy or size.

7. In figure S5A, images are shown that polarity is normal. However, it is also clear that the normal positions of daughter cells relative to each other at the 4 cell stage are significantly impacted. In Figure 5E, the P1 furrow is significantly mispositioned, is this due to changes in contacts between the cells? Given that Ran regulates the spindle, and the anaphase spindle elongation in AB drives the relative positions of daughter cells at the 4-cell stage, it concerns me that perhaps the spindle is abnormal in these cells. This also brings up the possibility that Ran affects the spindle, which more indirectly affects cytokinesis. Why is cell shape/overall positioning affected?

The reviewer makes an excellent point. It is important to note that we used mild depletion of RAN-3, and we only analyzed cells with proper chromosome segregation. However, as the reviewer points out, there appears to be a change in spindle position. In addition to the change in cytokinesis kinetics, we also see a specific delay in prometaphase in P1 cells. Because of this delay, the ABa and ABp cells have already rotated into position, causing the P1 cell to appear to divide away from contacts that would typically be visible in control cells. Strikingly, despite how division initiates, the P1 daughters (EMS and P2 cells) adopt ‘normal’ positions relative to the ABa and ABp cells, and P granules segregate into the P2 cells properly suggesting that their fates are properly established. To address concerns that the lower Ran-GTP levels could affect the spindle, we imaged tubulin in embryos depleted of RAN-3, and the centrosomes indeed appear to elongate similar to control cells (Fig. S7C).

8. It seems important to show that the Ran system affects anillin localization instead of only showing genetic interaction data.

Indeed, measuring the changes in ANI-1 localization after perturbing the Ran pathway is an important addition to the manuscript. As explained in the comments for reviewer 1, we have added ANI-1 localization data for control AB vs. P1, ran-3 RNAi and imb-1 RNAi (Figs 6C, E).

9. Several Ran players are depleted by RNAi, but the Ran gradient and/or localization and function of any individual Ran component is not directly studied in the manuscript. It would seem to be important to characterize how these players work in a direct way to understand how downstream targets might be affected.

We agree that it is important to look at the localization of Ran pathway components and the Ran gradient, especially given the interesting phenotypes observed for the Ran pathway components (e.g. depletion of IMB-1, IMA-3 and RAN-3). However, a good portion of this manuscript studies the differences in ring assembly between AB and P1 cells, and the new title suggested in point 1 will better reflect this. We are excited by the finding that the Ran pathway could account for the differences in ring assembly between AB and P1 cells, and we will pursue this by delving deeper into the mechanism by which the Ran pathway regulates ring assembly in a future study. Further, since the tools to study many of the Ran components do not currently exist for C. elegans, we need to generate new strains that would be beyond the scope of a revision.

Minor concerns:

10. In the intro, lines 95-98, Ran is proposed to enhance anillin recruitment to the cortex. However, when Ran regulates spindle assembly, it releases spindle factors from importin binding

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near the chromatin if I understand correctly. How can this pathway enhance anillin recruitment to the cortex if importins will be released from binding near chromatin?

We have clarified how importins can regulate the cortex in the text. For example, both our group and others observed the enrichment of importins at the cortex/membrane during mitosis in other organisms (e.g. Beaudet et al., 2017; Brownlee and Heald, 2019). In particular, the Brownlee and Heald paper showed that importin-alpha is palymitoylated, which may help with spindle scaling, but also could support cortical functions.

11. The finding that cell fate, size and Ran involvement has been previously described in other studies. It's not immediately clear how these results change or improve on what was previously known from the introduction.

We have added text to clarify this in the discussion. This includes how our findings build on knowledge from the Canman lab and their studies of cytokinesis in the 4-cell embryo, the Carvalho/Oegema labs and their studies of correlating size with constriction, and the studies from our lab describing the Ran regulation of cytokinesis in HeLa cells.

12. What has already been investigated regarding differences in cytokinesis regulation in different cell types in the embryo is not well described and compared with the new insights from this study.

The discussion has been edited to clarify the new findings from this study and how they relate to those from the Canman lab.

13. It is difficult to see the "myosin and actin levels were higher along the anterior cortex compared to the posterior" in Fig1E, perhaps arrows to highlight this? It is also hard to compare this with Fig 2E to see the difference.

Arrowheads have been added to Fig. 1E, and the line graphs were better aligned to help the readers visualize this change better in Fig. 2D (formerly 2E).

14. Line 349: How long was this RNAi depletion done, what was the percent cytokinesis failure under these conditions?

The RNAi conditions are described in the methods (e.g. 24 hours for ect-2 RNAi). We used the same timeframe for depletion for collecting embryos that succeeded vs. failed, as we were working close to the threshold for ect-2 requirement in AB and P1 cells (vs. germline and P0). It is difficult to calculate a percentage since we only filmed at this threshold (e.g. filming longer than this rapidly transitions to 100% failure, but also at P0). At this threshold, for example, we saw 3/16 AB or P1 cells fail during one imaging session, and overall the failure rate ranged between~ 0-20% due to variability in RNAi and depending on the filming session. We included ring closure for ect-2(RNAi) AB and P1 cells that fail cytokinesis (Fig. S6A) and characterized myosin filaments in these cells (Fig. S6B and E).

15. Line 457: I did not see the Ran experiment in figure S4.

This was an error and has been removed.

16. I do not understand what the finding that imb-1/ima-3 co-depletion means as it pertains to furrow ingression rate. Are they having opposite effects on the same targets, counteracting each other or something else?

We have altered the text to better clarify this for the reader. Since the co-depletions are only partial, it is difficult to interpret same vs. separate pathways per se, but it points to different threshold requirements for IMB-1 and IMA-3 pathway components in AB vs. P1 cells. We interpret this to mean that IMB-1 could more specifically regulate a target protein(s) such as ANI-1 in AB cells, while IMA-3 and/or the heterodimer does not vs. in P1 cells where IMA-3 and/or IMB-1 could regulate a target protein(s). Based on how importin-binding can impact protein function in other contexts, the heterodimer could cause different effects on binding (e.g. inhibition) vs. either on

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their own. However, this has not been studied for most targets, and we would not want to speculate based on so little biochemical data.

Second decision letter

MS ID#: JOCES/2021/258921

MS TITLE: Diverse mechanisms regulate contractile ring assembly for cytokinesis in the two-cell *C. elegans* embryo

AUTHORS: Imge Ozugergin, Karina Mastronardi, Christopher Law, and Alisa J Piekny
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

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

As you will see, the reviewers gave favourable reports but raised some critical points that will require amendments to your manuscript. I hope that you will be able to carry these out because I would like to be able to accept your paper, depending on further comments from reviewers.

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

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

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

Reviewer 1

Advance summary and potential significance to field

I apologize for the long delay. I thought I had taken care of this weeks ago.

The revision has addressed most of my concerns. Only a few issues remain:

Comments for the author

The authors focus on ring assembly, but only show kinetics of cell shape change.
This referee realizes that the term “ring assembly” has been assigned by the authors and others in the literature to a window of time, but it would be more clear to readers if the language of cortical remodeling and cytoskeletal activation and assembly (ring assembly) was kept separate from terms for cell shape change (furrow, doubled membrane, involution, and other terms).
All association of furrow asymmetry with cortical flows, which begins in the Introduction and continues into the text about Fig 1 (line 206) and subsequent conditions, should be removed unless

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the authors cite some evidence for a connection. Related: figure panels showing furrow asymmetry do not add anything to the Main figures and should be moved to supplement.
1D it is puzzling that AB and P1 samples ranged in size. Does this range reflect measurement error? The trends are slight or insignificant. It would seem that these results do not belong in Fig. 1 but rather in Fig 4 where the effect of cell size is tested, if they appear in the main text at all.
For area under the curve NMY-2 level data in Fig. 2F, variance (SD, SEM, or 95% CI) and the results of key statistical comparisons should be shown. Several comparisons are of interest. For example, anteriorizing vs posteriorizing the embryo w PAR-1 or -3 depletion has different effects on peak NMY-2 levels but similar effects on the shapes of the NMY-2 distribution curves. Is either of these two conditions significantly different from control AB (or control P1)?

Reviewer 2

Advance summary and potential significance to field

This is an interesting study and should have significance to the field.

Comments for the author

I have a few remaining points of clarification:
Regarding point 3: The additional data help clarify ingression dynamics in P1.
However, there is still a slight misrepresentation of AB furrow ingression. If one calculates the furrow asymmetry parameter similar to Maddox 2007 you get a very asymmetric value because the furrow never seems to ingress away from the AB/P1 cell boundary (this can be seen in Figure 1A). This allows the AB midbody to be inherited by EMS because it forms in contact with the neighboring cell.
This modified cytokinetic furrow behavior is particularly interesting in AB and implicates cell adhesion or other pathways controlling this behavior. The graphs as presented give the impression that the furrow ingresses away from the cell boundary. This point could be clarified in the text to avoid confusion with other studies (i.e. Bai et al., 2020).
Regarding point 10. It is interesting that importins can be in the cortex, but it is still confusing. The RAN gradient, as shown in Fig5A is low GTP in the cortex which won't promote release of cargo. The simple model is that importins bind and inhibit, which is reversed by RAN. In your response to point 16 you imply that importin binding might not be inhibitory? Perhaps this can be better explained as the generally understood pathway seems different than what is being implicated here.

Second revision

Author response to reviewers' comments

Reviewer 1 Comments for the author

1. The authors focus on ring assembly, but only show kinetics of cell shape change. This referee realizes that the term "ring assembly" has been assigned by the authors and others in the literature to a window of time, but it would be more clear to readers if the language of cortical remodeling and cytoskeletal activation and assembly (ring assembly) was kept separate from terms for cell shape change (furrow, doubled membrane, involution, and other terms).

Terms that are defined by time have been explicitly noted (i.e. as furrow initiation phase, ring assembly phase, constriction phase) to clarify for the reader that these refer to temporal vs. spatial events. We also explain early in the manuscript how the temporal phases were defined by spatial changes.

2. All association of furrow asymmetry with cortical flows, which begins in the Introduction and continues into the text about Fig 1 (line 206) and subsequent conditions, should be removed unless

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the authors cite some evidence for a connection. Related: figure panels showing furrow asymmetry do not add anything to the Main figures and should be moved to supplement.

We appreciate this suggestion and we have removed text linking cortical flows to asymmetric ring closure in the introduction and throughout the results section. We speculate on this relationship in the discussion, where it is clear to the reader that this is speculation vs. 'known'. Given this speculation and the novelty of measuring symmetry of closure in the different treatments for AB and P1 cells, we think it is valuable to include some of this data in the main text as there may be multiple factors contributing to this phenomenon.

3. 1D it is puzzling that AB and P1 samples ranged in size. Does this range reflect measurement error? The trends are slight or insignificant. It would seem that these results do not belong in Fig. 1 but rather in Fig 4 where the effect of cell size is tested, if they appear in the main text at all.

To demonstrate that the trends hold regardless of sample size, we added a supplemental figure showing that a smaller, random 'n' of P1 cells still shows correlation with duration of ring assembly and size.
We appreciate the suggestion to move panels relevant for size to Fig. 4. This has been done.

4. For area under the curve NMY-2 level data in Fig. 2F, variance (SD, SEM, or 95% CI) and the results of key statistical comparisons should be shown. Several comparisons are of interest. For example, anteriorizing vs posteriorizing the embryo w PAR-1 or -3 depletion has different effects on peak NMY-2 levels but similar effects on the shapes of the NMY-2 distribution curves. Is either of these two conditions significantly different from control AB (or control P1)?

We have added statistical analyses for area under the curve. We did not see significant differences for the accumulated peak myosin levels in cells after either of the PAR-depletions compared to control AB cells, but we did observe that PAR-1 depletion is significantly higher vs. control P1. This data supports that loss of the posterior PAR does lead to higher levels of myosin vs. depletion of the anterior PAR, but there seems to be a threshold that is reached is similar to AB control. Since AB-like myosin levels are found in both daughters for the PAR-depleted embryos vs. one (e.g. AB in control), this could explain the max. threshold.

Reviewer 2 Advance summary and potential significance to field

This is an interesting study and should have significance to the field.

Reviewer 2 Comments for the author

I have a few remaining points of clarification:

1. Regarding point 3: The additional data help clarify ingression dynamics in P1. However, there is still a slight misrepresentation of AB furrow ingression. If one calculates the furrow asymmetry parameter similar to Maddox 2007 you get a very asymmetric value because the furrow never seems to ingress away from the AB/P1 cell boundary (this can be seen in Figure 1A). This allows the AB midbody to be inherited by EMS because it forms in contact with the neighboring cell. This modified cytokinetic furrow behavior is particularly interesting in AB and implicates cell adhesion or other pathways controlling this behavior. The graphs as presented give the impression that the furrow ingresses away from the cell boundary. This point could be clarified in the text to avoid confusion with other studies (i.e. Bai et al., 2020).

The reviewer raises an excellent point and we have clarified the text to add the direction of asymmetry, and its noted importance in cell fate. We also included the Bai et al. 2020 reference to help support this description.

2. Regarding point 10. It is interesting that importins can be in the cortex, but it is still confusing. The RAN gradient, as shown in Fig5A is low GTP in the cortex, which won't promote release of cargo. The simple model is that importins bind and inhibit, which is reversed by RAN. In your response to point 16 you imply that importin binding might not be inhibitory? Perhaps this can be

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better explained as the generally understood pathway seems different than what is being implicated here.

We appreciate that the model is challenging, since we are proposing that importin-binding promotes vs. inhibits function of bound cargo. This is quite different compared to how Ran/importins have been shown to regulate spindle assembly. In the case of human anillin, we found that importin-binding helps stabilize an open conformation that makes the C-terminus accessible to lipid-binding. Importin-binding is required for anillin's cortical recruitment in anaphase, and loss of this binding can lead to cytokinesis defects. We have expanded our description of this model in the introduction in the context of the more conventional model to help readers follow the logic.

Third decision letter

MS ID#: JOCES/2021/258921

MS TITLE: Diverse mechanisms regulate contractile ring assembly for cytokinesis in the two-cell *C. elegans* embryo

AUTHORS: Imge Ozugergin, Karina Mastronardi, Christopher Law, and Alisa J Piekny
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

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