Cell polarity dependent centrosome separation in the C. elegans embryo

Alexandra Bondaz, Luca Cirillo, Patrick Meraldi, and Monica Gotta

Corresponding Author(s): Monica Gotta, University of Geneva and Patrick Meraldi, University of Geneva, Cell Physiology and Metabolism Dept.

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March 25, 2019

Re: JCB manuscript #201902109

Prof. Monica Gotta  
University of Geneva  
Cell Physiology and Metabolism  
1, Rue Michel Servet  
Geneva 4 1211  
Switzerland

Dear Monica -

I was asked to be the monitoring editor for your manuscript on "Cell Polarity Dependent Centrosome Separation in the C. elegans Embryo", and we have now received the reviews from three external referees with expertise in this area. I am pleased to be able to tell you that all of the referees considered the work to be interesting and insightful. However, they each bring up a number of points that will need to be addressed. Therefore, although we are unable to accept the manuscript in its present for publication, we would be pleased to consider a suitably revised version.

Both ref #1 and #3 suggest that instead of measuring timing of centrosome separation at a single point it would be much more informative to perform time courses so as to distinguish between a difference in the time at which separation is initiated versus a difference in the rate of separation. Ref#1 suggests trying to separate PAR asymmetry from PLK-1 asymmetry, if this is possible, and raises several other points about interpretation of phenotypes. Ref#2 recommends more detailed quantification of LIN-5 levels at the cortex, and asks if it is possible to image the phosphorylated form of PLK-1. They also suggest more discussion about the additional contributions of microtubules and to acknowledge that these may be obscured by some of the methods used for KLP-7 depletion. Ref#3 requests some insight into the biological consequences of KLP-7 regulated centrosome separation, and suggests looking at the ability of the cells to control chromosome congression and separation.

Overall, we feel that these points are addressable within the 3 months provided for revisions. Please note that together with a revised manuscript we will need a point-by-point response to each of the reviewer comments.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

Text limits: Character count for an Article is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

Figures: Articles may have up to 10 main text figures. Figures must be prepared according to the
The work by Bodaz et al uses the C. elegans embryo to explore the mechanisms of centrosome separation, which is important for proper spindle assembly and chromosome segregation. The manuscript clearly makes a number of interesting observations and their data support differences in the microtubule dynamics affecting centrosome separation. Specifically, they observe a cell type specific delay in the two-cell embryo, with the larger, somatic cell AB exhibiting a small delay in centrosome separation relative to its sister P1, and that this delay is strongly exacerbated by depletion of klp-7 / MCAK. They go on to show that this delay is linked to polarity, but not the fate of the two daughters, with data suggesting that the relative balance of PLK-1 distinguishes the response of the two cells through regulation of cortical LIN-5.
I do, however, have some reservations about the manuscript in its present form and I feel the authors could make a clearer case for their model, which was not entirely clear to me on first reading. My main concerns revolve around the link between the klp-7 phenotype and the normal delay observed between AB and P1 and the interpretation of the PLK-1 / LIN-5 results. My overall take is that AB is somehow more sensitive to changes in MCAK activity, which is interesting. This differential sensitivity likely arises due to polarity-dependent, but I might argue likely plk-1-independent, differences in pulling forces applied to centrosomes in the two cells, at least in wild-type embryos. This MCAK-depletion defect can be rescued by globally enhancing LIN-5 cortical localization by plk-1 RNAi, which presumably increases pulling forces to compensate for MCAK loss, though this treatment strikingly leaves asymmetry in LIN-5 intact. By contrast, the klp-7 mutant effect is exacerbated by reduced LIN-5/Galpha (e.g. lower pulling forces) which now renders P1 separation similarly sensitive to changes in MCAK activity as AB.

Major Comments:

(1) The difference between P1 and AB behavior is small, but significant in wild-type animals. However, the manuscript does not provide insight into how this delay is normally controlled, nor does it explore whether this is a generic property between germ and soma beyond the two-cell stage. Also, the PLK-1 pathway they explore seems to be specific to the klp-7 induced phenotype as it cannot explain the delay in wild type embryos. Does this suggest that klp-7 is a special case? How relevant are the results to the normal situation?

(2) The nature of the klp-7 phenotype is left unclear. The authors argue that it does not appear to be related to MT dynamics, but do not offer an alternative mechanism, leaving the model for how this all works somewhat muddled. In the Discussion, they suggest that astral microtubules may be different, but this seems to be contradicted by what they state in the Results. I would note that authors use the fact that klp-7 increases cortical MT density but not asymmetry in both AB and P1 to argue that MT density cannot explain the separation defects in AB. However, MT density in klp-7 P1 is roughly the same as WT AB, hence one might not expect separation defects in P1. By contrast, further increasing MT density in klp-7 AB could drive the cell across a critical threshold yielding a separation phenotype.

(3) The relevant role of PLK-1 appears to be to globally regulate LIN-5 levels. It is striking that par and mex mutants eliminate AB vs P1 differences, but plk-1 mutants do not. The authors use data that reduced, uniform levels of PLK-1 in par/mex are associated with loss of AB/P1 differences to argue for PLK-1 regulating the asymmetry in LIN-5 activity between the two cells. However, while PLK-1 levels become equivalent in par/mex conditions, it is unclear whether the conversion of P1 to an AB-like behaviour is due to this symmetric PLK-1 or loss of polarity per se. The fact that the difference between AB and P1 persists in plk-1 mutants would tend to make me believe that loss of polarity may be the dominant effect here. Is there a way to separate PLK-1 asymmetry from PAR asymmetry, for example, by disrupting binding of PLK-1 by MEX-5/6 as in Han et al (2018)? This would strongly support their model.

(4) The authors make a strong point that lin-5 is unique in its ability to rescue AB vs P1 differences. However, could the difference between lin-5 and Galpha be due to differences in penetrance? Galpha partially equalises AB and P1 separation in otherwise wild-type embryos, similar to lin-5. This effect is also clear in klp-7, as the P1 delay comes close to, although not quite matching AB. This would be consistent with a more simple model that reduced MCAK activity is simply compensated by increased cortical pulling forces. Interestingly, because lin-5 and Galpha reduce the difference
between AB and P1 in wild type, it suggests that differences in pulling forces are the root cause of
the difference in AB vs. P1. And that these are regulated by polarity, but not necessarily (or at least
not completely) by PLK-1 asymmetry. This would not undercut the result that plk-1 rescue of
separation in klp-7 acts via LIN-5, but would certainly simplify the model. I would predict that over-
expression of GPR1/2 (e.g. Redemann, 2011) might give a result similar to plk-1.

(5) The authors suggest that the major defect is the timing of centrosome separation. However,
generally, they only examine a single point. Can the authors distinguish between reduced
separation speed, delays in onset, and more general defects in separation? Does AB eventually
correct itself to reach normal separation? I also wonder whether timing information may give more
robust data compared to single data points? Could it be that differences in timing may be masked -
for example, the slower cell may catch up by the time the analysis is performed. I would
imagine this would be substantially more work and hence would only be worth doing if it provided
substantial benefit, but perhaps worth examining for a select dataset.

Other comments:

Statistics - The phenotypes seem quite variable. Hence lack of significant differences may in some
cases be due to low N. For example P1-control vs P1-klp7 is sometimes significant, sometimes not.

PLK-1 levels are examined using an endogenously tagged transgene, which is likely a improvement
over prior immunostainings. However, tagging a protein could on its own interfere with normal
degradation. A western would be preferable if antibodies were available.

Figure S1D - JDU316 with no depletion - I assume they mean to stress that this is a mutant rather
than RNAi, but the phrasing is a bit unclear.

Does klp-7 alter LIN-5 levels/localization?

Have the authors attempted to measure differences in pulling forces in the two cells? Do
centrosomes in 2/4-cell embryos show similar differences in they way they disassemble as do
anterior and posterior centrosomes which would be suggestive of the differences implied by this
work?

Reviewer #2 (Comments to the Authors (Required)):

In the manuscript by Bondaz et al. entitled "Cell polarity dependent centrosome separation in the C.
elegans embryo" the authors observe that centrosome separation is always slower in the AB cell
versus the P1 cell in the early cell divisions. This is significant because delayed centrosome
separation is known to interfere with the fidelity of chromosome segregation. Thus, it is an
important phenomenon for further investigation. Loss of KLP-7 exacerbated this observation which
is broadly consistent with previous work from Tanenbaum et al (2009) showing that loss of the
orthologue MCAK/Kif2C interfered with bipolar spindle assembly. The authors investigate the
mechanism for this relationship by revealing that PLK-1 enrichment in the AB cell, rather than MT
dynamics per se, is the key event that slows centrosome separation in the AB cell, specifically. The
authors use individual and combinations of siRNA treatments and thermosensitive mutants to
identify key components responsible for centrosome separation by studying the AB cell which
exhibits impaired centrosome separation in the absence of klp-7 due to higher levels of plk1 in
those cells. The downstream target of plk1 appears to be lin-5 which is responsible for coupling
force generating components to the astral microtubules. This is consistent with work from the Cheeseman lab showing that centrosomal polo kinase impacts the establishment of cortical force generators in mammalian cells.

The authors have convincingly shown that depletion of klp-7 suppresses centrosome separation but only in the AB cell. This is an interesting study because, in my opinion, the detailed mechanistic underpinnings for centrosome separation are understudied and dominated by Eg5. I consider the experiments sufficient to support their main conclusions and sufficient to further the field. For these reasons I strongly support the publication of this manuscript in the JCB.

Below are listed comments, rather than criticisms of the manuscript that the authors may want to address:

1- The text is written as if to discard the mechanistic contribution of changes MT dynamics as an inhibitor of centrosome separation, however, the authors have clearly shown that it impacts centrosome separation in both AB and P1 - at least in Figure 1B (although the effect is variable in repeated experiments). Thus, while longer denser MTs may be correlated with an antagonistic effect on centrosome separation, this correlation is not the explanation for the difference between AB and P1. I would make the effort to make sure that this is clear in the text because the effect of the klp-7 background is interesting and the data are nicely quantified.

2- It has been reported by the Bowerman lab that maternal tubulin is autoregulated. Longer and denser microtubules reported by the authors in the absence of klp-7 suggests that tubulin synthesis is increased and one can't help but wonder if this could lead to different results for RNA interference depletion versus thermosensitive strains. Comparisons between depletion and or1092ts could be used to distinguish between effects arising from excess tubulin synthesis. This is not to say that the basic premise: that klp-7 loss suppresses centrosome separation, is in question. All the methods used for klp-7 depletion presented in this manuscript support this. But details, of the mechanism might be different between these depletion methods. I am not requiring that these experiments be added because MT dynamics changes are not responsible for the differential response of AB which is the topic of the investigation, however, it is a subject worthy of discussion.

3- The authors have convincingly shown that plk1 kinase is distributed throughout the cell and higher in level in AB. However, this may not reflect the level of activated plk1 (in mammalian cells this is usually indicated using anti-phospho antibodies that detect an activating phosphorylation event). Do such reagents exist for use in C. elegans? Do the authors have reason to suspect that the inhibitory effect of active plk1 is emanating from the centrosome as has been shown in mammalian cells?

4- A feature of the establishment of cortical pulling machinery is that it is dynamic and not uniform along the cortex. Two line scans (as described in the Methods) might not be sufficient to quantify this parameter. The reproducibility would depend on the X,Y and Z position within the embryo. Could the authors use CellProfiler, for example, to process an entire Z-stack and quantify the fluorescence LIN-5 a few pixels on either side of the cortex?

Reviewer #3 (Comments to the Authors (Required)):

The manuscript by Bondaz et al. studied the dynamics of centrosome separation in C. elegans embryos. They find that at the 2-cell stage centrosome separation at mitotic entry is slightly smaller
in the anterior AB cell compared to the posterior P1 cell. Depletion of the MCAK ortholog klp-7 does not affect separation in P1 but strongly decreases centrosome separation in AB. They show that this is not due to differences in microtubule dynamics or cell size but rather depends on PAR protein-dependent cytoplasmic polarity. The mitotic regulator plk-1 is enriched in the AB cell compared to the P1 cell, and the authors find that impairing plk-1 function rescues the klp-7-dependent defect and equalizing plk-1 by depletion of mex-5/6 induces this defect in P1. Finally they show that efficient centrosome separation in klp-7-depleted P1 cells requires the NuMa ortholog lin-5 and that cortical lin-5 levels are increased in both cells, but more drastically in AB, when plk-1 is depleted. This leads them to propose a model in which PAR protein-dependent accumulation of plk-1 in the anterior leads to a decrease in lin-5 levels, which impairs centrosome separation when klp-7 is depleted.

Overall the work is original, the analyses are well done and the manuscript contains many results that describe an interesting phenomenon that should broadly appeal to readers interested in mitotic regulation. I have a few points that should be considered to strengthen the work.

1. My main point is regarding the biological significance of the findings. The centrosome separation defect that is observed upon depletion of klp-7 is clear and robust, yet it is not clear from the manuscript results whether this is relevant for mitosis. Do the authors observe differences in mitotic timing, chromosome congression and/or chromosome segregation in AB vs P1 after klp-7 depletion? If not, is this because of redundancy and what would ensure this redundancy? Is mitosis in AB more sensitive to microtubule poisons than P1, with or without klp-7 depletion? I think that anchoring the observed centrosome separation defect into the biology of embryonic development would be desirable.

2. Also, the authors report on centrosome separation at a single timepoint, i.e. NEBD, but the analysis could go a little further to better shed light on the mechanism of regulation. More specifically I think that it could be interesting to assess whether the difference in centrosome separation observed between AB and P1 in klp-7-depleted embryos is due to a difference in the timing of separation initiation (which would be linked to the cell cycle per se) and/or the speed of separation (which would relate to the regulation of cortical motor activity). I suspect the latter but it would be nice to confirm it. Acquiring such parameters could contribute to shed additional light on the mechanism at play.

Minor points:

3. In regards to the germline vs somatic lineage aspect of the model that is proposed in the discussion, the authors propose that germ cells have more robust mechanisms to ensure proper spindle assembly than somatic cells. They could perhaps consider testing this hypothesis, for instance by assessing whether the observed difference in centrosome separation that they see in AB and P1 is also present in the daughters of P1, with or without klp-7. If this is a germline vs soma phenotype the effect should be seen in EMS and not in P2.

4. Lines 244-245: I suggest being more precise on what is the effect of lin-5 impairment in klp-7-depleted embryos, i.e. state that it does not affect separation in AB but impairs separation in P1.

5. Lines 250 & 252: lin-5 is not written properly.

6. Last experimental section: The authors state that they used a lin-5::gfp strain from Heppert et al. In this paper however lin-5 was tagged with mNeonGreen. If this is the same strain, please correct
the genotype here are everywhere else in the manuscript (it is sometimes entered at lin-5::gfp and sometimes as lin-5::nNG), otherwise please reference the correct paper.

7. In this same section, I think that it would be essential to provide a few more details on how the measurements of lin-5 cortical intensity were made. For instance, were all cells roughly at the same cell cycle stage or were measurements done at different stages? Depending on how the acquisitions were done, differences in photobleaching and/or cell cycle stage could impact values obtained.

8. Lines 403-404: Please indicate concentrations rather than absolute amounts for the Mowiol solution. Same in line 411.

9. Lines 413-415: Please move these lines into the "Image acquisition and analysis" section. Also for all acquisitions described in this section please specify the fluorescence illumination source and the duration of illumination.

10. Table S2: Please cite the correct reference for strain TH32 (I think that it is Oegema K, et al. (2001) JCB 153:1209-1226).
Point-by-point response:

We thank all reviewers for their constructive comments which we have addressed with new experiments, better quantifications and better explanations in the text.

Below are the detailed answers to each comment.

Reviewer #1 (Comments to the Authors (Required)):

Major Comments:

(1) The difference between P1 and AB behavior is small, but significant in wild-type animals. However, the manuscript does not provide insight into how this delay is normally controlled, nor does it explore whether this is a generic property between germ and soma beyond the two-cell stage. Also, the PLK-1 pathway they explore seems to be specific to the klp-7 induced phenotype as it cannot explain the delay in wild type embryos. Does this suggest that klp-7 is a special case? How relevant are the results to the normal situation?

The observation of the reviewer that Plk1 only rescues the klp-7 phenotype is correct, but at this stage one cannot conclude that the difference seen in wild-type animals does not depend on Plk1, as it is not possible to fully deplete Plk1. We can only conclude that partial Plk1 inactivation is sufficient to rescue the klp-7 phenotype, which we interpret as a sensitized background, and is not sufficient to equalize AB and P1 in wild-type animals. We now make these assumptions more explicit in the discussion (page 16, lines 335-340).

With regard to question as to whether our observations are valid beyond the two-cell stage, we have now, as also requested by reviewer 3, analyzed centrosome separation in klp-7 mutants at the 4-cell stage and find a consistent pattern: centrosome at NEBD are less separated in ABa and ABp cells when compared to P2. We also note that in EMS the extent of centrosome separation is more variable than in P2, although the median is not statistically different. Overall, this implies that our findings are of more generic nature, beyond the 2-cell stage. These data are presented in the text (page 7, lines 113-119 and in Supplementary figure S1E).

(2) The nature of the klp-7 phenotype is left unclear. The authors argue that it does not appear to be related to MT dynamics, but do not offer an alternative mechanism, leaving the model for how this all works somewhat muddled. In the Discussion, they suggest that astral microtubules may be different, but this seems to be contradicted by what they state in the Results. I would note that authors use the fact that klp-7 increases cortical MT density but not asymmetry in both AB and P1 to argue that MT density cannot explain the separation defects in AB. However, MT density in klp-7 P1 is roughly the same as WT AB, hence one might not expect separation defects in P1. By contrast, further increasing MT density in klp-7 AB could drive the cell across a critical threshold yielding a separation phenotype.

We agree with the reviewers that this point should have been better explained. First, our goal was not to state the klp-7 phenotype is unrelated to microtubule dynamics, but that the difference in klp-7 AB vs P1 cells cannot be explained by a differential regulation of microtubule dynamics by KLP-7 in AB vs P1. The physical separation of the centrosomes reflects the sum of the forces acting on centrosomes. We now more explicitly postulate in our discussion that klp-7 depletion increases the forces holding the two centrosomes together in both cells via its control of microtubule dynamics, but that these forces are selectively overcome by LIN-5 dependent cortical pulling forces in P1 (page 16-17, lines 350-367).

Second, our new analysis of centrosome separation over the whole 2-cell stage cycle in AB and P1 reveals important aspects on the mechanisms by which KLP-7 depletion impairs centrosome separation. Depletion of KLP-7 does not slow down the centrosome separation rate in AB when compared to P1, but instead delays the initial start of centrosome separation. In other words,
KLP-7 only affects the two centrosomes when they are very close to each other. This suggests that KLP-7 might prevent the formation of inter-polar microtubules that could be hold together by bridging proteins, such as kinesins-14 or SPD-1. These hypotheses are now explicitly discussed in the new discussion (page 16-17, lines 350-367).

(3) The relevant role of PLK-1 appears to be to globally regulate LIN-5 levels. It is striking that par and mex mutants eliminate AB vs P1 differences, but plk-1 mutants do not. The authors use data that reduced, uniform levels of PLK-1 in par/mex are associated with loss of AB/P1 differences to argue for PLK-1 regulating the asymmetry in LIN-5 activity between the two cells. However, while PLK-1 levels become equivalent in par/mex conditions, it is unclear whether the conversion of P1 to an AB-like behaviour is due to this symmetric PLK-1 or loss of polarity per se. The fact that the difference between AB and P1 persists in plk-1 mutants would tend to make me believe that loss of polarity may be the dominant effect here. Is there a way to separate PLK-1 asymmetry from PAR asymmetry, for example, by disrupting binding of PLK-1 by MEX-5/6 as in Han et al (2018)? This would strongly support their model.

Previous studies have established that the anterior and posterior PAR protein distribution is maintained in MEX-5/6 depleted embryos (Schubert et al., 2000, Cuenca et al., 2003, Cheeks et al., 2004). We have now made this clear in the text and referenced the relevant literature in page 12, lines 242-245). In the Han et al. paper Threonine(T186) of MEX-5 has been mutated to Alanine. This threonine is a polo docking site which, after being phosphorylated, binds to PLK-1 (Nishi et al., 2008). However, in the single mex-5(T186A) mutant, the PLK-1 anterior gradient is maintained (our unpublished observation) most likely due to a redundant function provided by MEX-6. RNAi of MEX-6 in this strain leads to 100% lethality (our data and Han et al., 2018) and loss of the PLK-1 gradient. As in the MEX-5/6 depletion, PAR polarity is maintained.

(4) The authors make a strong point that lin-5 is unique in its ability to rescue AB vs P1 differences. However, could the difference between lin-5 and Galpha be due to differences in penetrance? Galpha partially equalises AB and P1 separation in otherwise wild-type embryos, similar to lin-5. This effect is also clear in klp-7, as the P1 delay comes close to, although not quite matching AB. This would be consistent with a more simple model that reduced MCAK activity is simply compensated by increased cortical pulling forces. Interestingly, because lin-5 and Galpha reduce the difference between AB and P1 in wild type, it suggests that differences in pulling forces are the root cause of the difference in AB vs. P1. And that these are regulated by polarity, but not necessarily (or at least not completely) by PLK-1 asymmetry. This would not undercut the result that plk-1 rescue of separation in klp-7 acts via LIN-5, but would certainly simplify the model. I would predict that over-expression of GPR1/2 (e.g. Redemann, 2011) might give a result similar to plk-1.

We thank the reviewer for suggesting an alternative hypothesis. The expression of GPR-1 in the strains published in Redemann et al., 2011 is unfortunately silenced. Following the suggestion of Henrik Bringmann, we have requested a new strain (Artiles et al., 2019). Unfortunately, we were not able to test the effects of GPR1/2 overexpression, as it leads to the formation of double nuclei in both AB and P1, with the centrosomes sandwiched between the two nuclei. This prevents our ability to track centrosome separation in a normal context.

Our data suggest that the difference in results seen after Galpha or lin-5 impairment is not due to a difference in penetrance. Indeed, when we used size asymmetry as a read-out, we observed that Galpha depletion resulted in 2 equal cells, whilst the lin-5(ts) mutant we used still retained a partial asymmetry in size (page 19, lines 404-408). This suggests, that if at all, Galpha depletion is more penetrant. We now mention this possibility in the discussion in a more explicit manner (page 17, lines 369-378).

(5) The authors suggest that the major defect is the timing of centrosome separation. However, generally, they only examine a single point. Can the authors distinguish between reduced separation speed, delays in onset, and more general defects in separation? Does AB eventually
correct itself to reach normal separation? I also wonder whether timing information may give more robust data compared to single data points? Could it be that differences in timing may be masked - for example, the slower cell may catch up by the timepoint the analysis is performed. I would imagine this would be substantially more work and hence would only be worth doing if it provided substantial benefit, but perhaps worth examining for a select dataset.

We thank the reviewer for this suggestion. We have now followed centrosome separation over time. This revealed two key points:

1. Centrosome separation relative to NEBD is not more efficient in P1 cells because they enter mitosis later, as we find in klp-7 mutants that the two centrosomes in P1 are usually already well separated when the nuclear envelope breaks down in AB (see new Supplementary Movies 1-3).

2. We find that klp-7 loss does not affect the rate of centrosome separation, but instead prevents the initial separation of the two centrosomes, suggesting that in the absence of KLP-7 the two centrosomes fail to overcome an initial force threshold that holds them together. (See Figure 2D)

Other comments:

Statistics - The phenotypes seem quite variable. Hence lack of significant differences may in some cases be due to low N. For example P1-control vs P1-klp7 is sometimes significant, sometimes not.

We agree with the reviewer that all these experiments are inherently variable to some extent, and that differences must be interpreted with care. Importantly, whereas a significant difference allows to exclude the null hypothesis (the two conditions behave the same), lack of a significant difference does not prove that the two conditions are the same. For this reason we interpreted our experiment conservatively, and tried to only draw conclusions when the effects were strong and reproducible. The one borderline case is the case of P1 control vs P1 klp-7, which in most but not all experimental series is statistically different, which is exactly the type of pattern one would expect to see in case of a weak but consistent difference. To make the reader aware of this, we now specifically point to this in the discussion (page 17, line 363-366).

PLK-1 levels are examined using an endogenously tagged transgene, which is likely an improvement over prior immunostainings. However, tagging a protein could on its own interfere with normal degradation. A western would be preferable if antibodies were available.

Unfortunately, we cannot use immunoblotting to compare protein levels in AB and P1 cells, as it is not possible to separate the two cell populations from embryos (in an amount that is sufficient for this kind of analysis). Fluorescence microscopy is the only methodology allowing a direct comparison within the same embryo.

Figure S1D - JDU316 with no depletion - I assume they mean to stress that this is a mutant rather than RNAi, but the phrasing is a bit unclear.

We thank the reviewer for pointing this out and have now improved our labelling.

Does klp-7 alter LIN-5 levels/localization?

As suggested by the reviewer we quantified LIN-5 levels in wild-type and klp-7 animals and found no difference (Figure S3B, C and D).

Have the authors attempted to measure differences in pulling forces in the two cells? Do centrosomes in 2/4-cell embryos show similar differences in the way they disassemble as do anterior and posterior centrosomes which would be suggestive of the differences implied by this work?
We have not attempted to measure forces in 2 cell-embryos. In these embryos, the AB spindle forms perpendicular to the plane of the microscope, making such experiments difficult.

Reviewer #2 (Comments to the Authors (Required)):

Below are listed comments, rather than criticisms of the manuscript that the authors may want to address:

1- The text is written as if to discard the mechanistic contribution of changes MT dynamics as an inhibitor of centrosome separation, however, the authors have clearly shown that it impacts centrosome separation in both AB and P1 - at least in Figure 1B (although the effect is variable in repeated experiments). Thus, while longer denser MTs may be correlated with an antagonistic effect on centrosome separation, this correlation is not the explanation for the difference between AB and P1. I would make the effort to make sure that this is clear in the text because the effect of the klp-7 background is interesting and the data are nicely quantified.

We agree with this reviewer that this point could have be better explained, which we now did in our new discussion. See also point 2 of reviewer 1.

2- It has been reported by the Bowerman lab that maternal tubulin is autoregulated. Longer and denser microtubules reported by the authors in the absence of klp-7 suggests that tubulin synthesis is increased and one can't help but wonder if this could lead to different results for RNA interference depletion versus thermosensitive strains. Comparisons between depletion and or1092ts could be used to distinguish between effects arising from excess tubulin synthesis. This is not to say that the basic premise: that klp-7 loss suppresses centrosome separation, is in question. All the methods used for klp-7 depletion presented in this manuscript support this. But details, of the mechanism might be different between these depletion methods. I am not requiring that these experiments be added because MT dynamics changes are not responsible for the differential response of AB which is the topic of the investigation, however, it is a subject worthy of discussion.

We think that the primary reason for the longer and denser microtubules in the absence of KLP-7, is that KLP-7 is a well-established plus-end directed microtubule depolymerase that restricts the growth of astral microtubules. While we agree with reviewer 2 that in addition tubulin auto-regulation might play a role, which would be very interesting, at this stage this would be highly speculative and would require us to introduce in the discussion the whole concept of tubulin auto-regulation, which lies outside of the main storyline. We therefore prefer to not include this discussion point.

3- The authors have convincingly shown that plk1 kinase is distributed throughout the cell and higher in level in AB. However, this may not reflect the level of activated plk1 (in mammalian cells this is usually indicated using anti-phospho antibodies that detect an activating phosphorylation event). Do such reagents exist for use in C. elegans? Do the authors have reason to suspect that the inhibitory effect of active plk1 is emanating from the centrosome as has been shown in mammalian cells?

Unfortunately, antibodies for activated C. elegans PLK-1 do not exist. We have attempted to raise such antibodies in the past, but with no success. We and many other laboratories have also extensively tried antibodies raised against human activated Plk1 in C. elegans. Whereas in our hands these antibodies work on a western blot (Tavernier et al., 2013), we never had consistent and clean results in stainings. In short, we observe a signal but this signal is not always gone when we deplete PLK-1 and does not change when we deplete SPAT-1 (Bora) and AIR-1.
(Aurora A), both required to activate PLK-1 in vitro and human Plk1 in vivo.

4-A feature of the establishment of cortical pulling machinery is that it is dynamic and not uniform along the cortex. Two line scans (as described in the Methods) might not be sufficient to quantify this parameter. The reproducibility would depend on the X,Y and Z position within the embryo. Could the authors use CellProfiler, for example, to process an entire Z-stack and quantify the fluorescence LIN-5 a few pixels on either side of the cortex?

As also requested by reviewer 3, we have now quantified the data in a second, complementary manner, which quantifies LIN-5 along a larger area of the AB and P1 cortices and we have added this analysis in Figure 7. Importantly, this novel analysis confirms our initial observation that Plk1 depletion increases cortical LIN-5 levels, and that this effect is particularly prominent in the AB cell.

The method of quantification has been added to material and methods as follows:

“LIN-5 levels were measured on 2-cell embryos (at the stage shown in figure 7) using two methods: to obtain the line profile of cortical proteins shown in Figure 7B, a 10-pixel-wide x 3 μm-long line scan overlapping the cortex was used on the maximum projection of the 5 planes. For each embryo the average of 2 line scans in AB and P1 was used for quantification. The line profile of individuals cells were normalized to the maximum value of wild-type AB cells. For the quantification shown in Figure 7C, we created an ImageJ macro to calculate the mean gray value of a 16-μm long line profile (https://github.com/LCirillo/FijiMacro/blob/master/IJ_Macro_CorticalLin5). Briefly, after background subtraction and maximum projection of 5 z planes (0.5 μm step size), a 10 px wide line was manually traced around the embryo cortex for both AB and P1. The point by point mean gray value was then averaged for a distance that spanned 8 μm form the center of the line profile, in both directions. The value obtained was normalized on the cytoplasmic mean gray value of the corresponding cell, calculated on the same image, using a 90x90 px square. The data were analyzed using Python 3.7.0 and Prims 8 (GraphPad)”

Reviewer #3 (Comments to the Authors (Required)):

1. My main point is regarding the biological significance of the findings. The centrosome separation defect that is observed upon depletion of klp-7 is clear and robust, yet it is not clear from the manuscript results whether this is relevant for mitosis. Do the authors observe differences in mitotic timing, chromosome congression and/or chromosome segregation in AB vs P1 after klp-7 depletion? If not, is this because of redundancy and what would ensure this redundancy? Is mitosis in AB more sensitive to microtubule poisons than P1, with or without klp-7 depletion? I think that anchoring the observed centrosome separation defect into the biology of embryonic development would be desirable.

To address this point, we monitored chromosome segregation using a strain expressing a histone::GFP transgene. Within the resolution of confocal spinning-disk microscopy we could, however, not observe a higher incidence of chromosome segregation defects or other types of defects in the AB cell when compared to P1. Since the spindle in AB is often perpendicular to the plane of imaging, it is difficult to follow chromosome congression. We conclude that the centrosome separation defect does not lead to major segregation defects, but cannot exclude minor defects. It is also worth noting that lack of KLP-7 is associated with a high embryonic lethality, but whether this phenotype is caused by the centrosome separation defects cannot be determine at this stage. We now discuss these points in the manuscript (page 16, lines 343-349).

As requested by this reviewer, we measured the timing of klp-7 mutant embryos (Figure below). In short, The AB cell cycle does not significantly change, while P1 becomes faster.
2. Also, the authors report on centrosome separation at a single timepoint, i.e. NEBD, but the analysis could go a little further to better shed light on the mechanism of regulation. More specifically I think that it could be interesting to assess whether the difference in centrosome separation observed between AB and P1 in klp-7-depleted embryos is due to a difference in the timing of separation initiation (which would be linked to the cell cycle per se) and/or the speed of separation (which would relate to the regulation of cortical motor activity). I suspect the latter but it would be nice to confirm it. Acquiring such parameters could contribute to shed additional light on the mechanism at play.

As requested, we have now added data (Figure 2D and 4D) where we follow centrosome separation over time. This has allowed us to conclude that klp-7 loss does not affect centrosome separation rates, but impairs the initiation of centrosome separation (see also point 5 of reviewer 1)

Minor points:

3. In regards to the germline vs somatic lineage aspect of the model that is proposed in the discussion, the authors propose that germ cells have more robust mechanisms to ensure proper spindle assembly than somatic cells. They could perhaps consider testing this hypothesis, for instance by assessing whether the observed difference in centrosome separation that they see in AB and P1 is also present in the daughters of P1, with or without klp-7. If this is a germline vs soma phenotype the effect should be seen in EMS and not in P2.

As suggested by this reviewer and reviewer 1, we have now looked at later embryos (Figure S1) and we observe that the difference is maintained and that centrosomes separate better in the P lineage (see also point 1 of reviewer 1).

4. Lines 244-245: I suggest being more precise on what is the effect of lin-5 impairment in klp-7-depleted embryos, i.e. state that it does not affect separation in AB but impairs separation in P1.

We thank the reviewer for this comment and correct this point accordingly

5. Lines 250 & 252: lin-5 is not written properly.
We thank the reviewer for pointing out this error.

6. Last experimental section: The authors state that they used a lin-5::gfp strain from Heppert et al. In this paper however lin-5 was tagged with mNeonGreen. If this is the same strain, please correct the genotype here and everywhere else in the manuscript (it is sometimes entered at lin-5::gfp and sometimes as lin-5::nNG), otherwise please reference the correct paper.

We thank the reviewer for spotting this error. This is the LIN-5 Neon green line. The error has been corrected.

7. In this same section, I think that it would be essential to provide a few more details on how the measurements of lin-5 cortical intensity were made. For instance, were all cells roughly at the same cell cycle stage or were measurements done at different stages? Depending on how the acquisitions were done, differences in photobleaching and/or cell cycle stage could impact values obtained.

The embryos were all roughly at the same stage which corresponds to the stage shown in the figure. The imaging conditions were the same for all embryos. As also requested by reviewer 2 we have now quantified the data in an additional manner. Please see point 4 of reviewer 2, Figure 7 and Material and methods, page 23-24.

8. Lines 403-404: Please indicate concentrations rather than absolute amounts for the Mowiol solution. Same in line 411.

This has been corrected

9. Lines 413-415: Please move these lines into the "Image acquisition and analysis" section. Also for all acquisitions described in this section please specify the fluorescence illumination source and the duration of illumination.

We have moved this part and added the missing information.

10. Table S2: Please cite the correct reference for strain TH32 (I think that it is Oegema K, et al. (2001) JCB 153:1209-1226).

Thank you for pointing this out.
July 25, 2019

Re: JCB manuscript #201902109R

Prof. Monica Gotta
University of Geneva
Cell Physiology and Metabolism
1, Rue Michel Servet
Geneva 4 1211
Switzerland

Dear Prof. Gotta,

Thank you for submitting your revised manuscript entitled "Cell polarity dependent centrosome separation in the C. elegans embryo". The manuscript has been seen by the original reviewers whose full comments are appended below. While the reviewers continue to be overall positive about the work in terms of its suitability for JCB, some important issues remain.

You will see that we have positive responses from two reviewers (including Rev#2 in first round). However, Reviewer #1 has some concerns with whether or not Klp7 has a role in centrosome separation that occurs in normal polarized cells, and the underlying mechanisms in general, but perhaps does not clearly explain how they might be resolved. We feel that there is no need for further experimental work, but the Discussion could be modified to more clearly explain the model and how the new data fit into it, and to address the additional points raised by Reviewer #1 where appropriate.

- Please provide a short eTOC blurb
- Add a paragraph after the Materials and Methods section briefly summarizing the online supplementary details
- Add conflict of interest statement to the Acknowledgements section
- Provide tables as excel files
- Provide main and supplementary text as separate, editable .doc or .docx files

Our general policy is that papers are considered through only one revision cycle; however, given that the suggested changes are relatively minor we are open to one additional short round of revision. Please note that I will expect to make a final decision without additional reviewer input upon resubmission.

Please submit the final revision within one month, along with a cover letter that includes a point by point response to the remaining reviewer comments.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact me or the scientific editor listed below at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.
Reviewer #1 (Comments to the Authors (Required)):

In their revised manuscript, the authors continue to provide interesting new observations based on careful experimentation regarding cell-type specific regulation of centrosome separation, which is certainly of general interest and potentially suitable for JCB. The authors have also addressed a number of my technical concerns. However, my major reservations of the previous version remain. I would have liked to see more clear insight into the mechanisms behind the role of klp-7 in centrosome separation, whether the klp-7 delay phenotype is related to the mechanism of delay in wild-type animals, and/or how the klp-7 rescue by plk-1 is relevant to the WT case. I would echo comments from Reviewer 3 that it would benefit this work to more tightly relate the phenomenology and significance to the biology of the embryo.

Perhaps my feelings are due in part to the fact that despite some very interesting data, there are two, somewhat incomplete, stories here. One story relates to the role of klp-7 in facilitating centrosome separation, which highlights the complex balance of forces at work in separation, but which is not really explored at the mechanistic level. The second explores the role of PLK-1 in creating a delay between AB and P1, which is linked to PLK-1 asymmetry and its effects on LIN-5, but the significance of which remains unclear. Because klp-7 is so prominent, it raises many interesting questions regarding mechanism and force balance in the context of how the klp-7 delay arises and how it is rescued, but answers are largely left to speculation. At the same time the relevance of the mechanism to the WT context is not made clear.

With regard to the polarity-dependent delay, the necessity of partial depletion of PLK-1 does limit the possible conclusions. It is notable that the AB-P1 delay and corresponding LIN-5 asymmetry remain intact in plk-1(RNAi) in both WT and klp-7 backgrounds, consistent with residual PLK-1 activity, but this could also reflect an underlying PLK-1-independent asymmetry, leaving open the degree to which PLK-1 is required in WT for the delay. More clear is the effect of symmetric PLK-1 on the loss of the AB-P1 difference. It would be nice to confirm in their experiments that T186-MEX-5 plus mex-6(RNAi) reproduces the delay and symmetric LIN-5 localization to eliminate roles for other upstream cues. This would be relatively convincing in terms of how the delay is achieved, but does not really address whether the observed delays are important to the embryo.

The new temporal data is a good addition. However, it reveals a rather curious relationship between timing of separation onset and separation speed. AB separates later, but faster than P1. Similarly, klp-7 separates later, but faster than WT. Rather surprisingly, the postulated increase in pulling forces (plk-1) seems to change timing, but not speed of separation. Is it reasonable to expect that overcoming the intracentrosomal connections would depend on the magnitude of cortical pulling forces but the velocity of separation does not? Again, there is interesting data here, but it is not
really discussed or explored further. I find it these results difficult to reconcile with the proposed model.

Minor comments:
Line 166 - "KLP-7 specifically delays the initiation of centrosome separation in AB." I would argue that klp-7 delays both, albeit AB moreso, but also accelerates separation speed such that P1 largely catches up by NEBD.

Western blots - While AB vs P1 westerns are not possible, par-2 vs par-3 would be relevant here. I don't feel this is necessary to address, but would confirm their conclusions versus prior observations.

Reviewer #3 (Comments to the Authors (Required)):

The resubmitted manuscript by Bondaz et al. is significantly improved from the initial submission and I consider that the authors have done an excellent job to address my suggestions as well as those of the other two reviewers. The work is careful and the conclusions, as stated, are well supported by the data. I consider that the work will appeal to JCB readers interested in the coupling between cell polarity and aspects of cell cycle regulation during embryonic development and I am therefore highly supportive of its publication.
We thank the reviewers for their constructive comments. We are pleased that reviewer 2 and 3 recommend the publication of our manuscript and have addressed the remaining comments of reviewer 1 in the following manner:

Major Comments:

(1) In their revised manuscript, the authors continue to provide interesting new observations based on careful experimentation regarding cell-type specific regulation of centrosome separation, which is certainly of general interest and potentially suitable for JCB. The authors have also addressed a number of my technical concerns. However, my major reservations of the previous version remain. I would have liked to see more clear insight into the mechanisms behind the role of klp-7 in centrosome separation, whether the klp-7 delay phenotype is related to the mechanism of delay in wild-type animals, and/or how the kpl-7 rescue by plk-1 is relevant to the WT case. I would echo comments from Reviewer 3 that it would benefit this work to more tightly relate the phenomenology and significance to the biology of the embryo.

Perhaps my feelings are due in part to the fact that despite some very interesting data, there are two, somewhat incomplete, stories here. One story relates to the role of klp-7 in facilitating centrosome separation, which highlights the complex balance of forces at work in separation, but which is not really explored at the mechanistic level. The second explores the role of PLK-1 in creating a delay between AB and P1, which is linked to PLK-1 asymmetry and its effects on LIN-5, but the significance of which remains unclear. Because klp-7 is so prominent, it raises many interesting questions regarding mechanism and force balance in the context of how the klp-7 delay arises and how it is rescued, but answers are largely left to speculation. At the same time the relevance of the mechanism to the WT context is not made clear.

Previous studies had implicated MCAK, the mammalian orthologue of KLP-7, in the establishment (van Heesbeen et al. Chromosoma 2017) and maintenance of spindle bipolarity during prometaphase and when Eg5 activity is impaired (Kollu et al., Curr Biol. 2009; Tanenbaum et al., Curr. Biol 2009). Nevertheless, to our knowledge this is the first study linking KLP-7/MCAK to centrosome separation before nuclear envelope breakdown and in the absence of other sensitizing backgrounds (such as Eg5 inhibition). Moreover, our new data demonstrates that MCAK plays a critical role in the initial separation of centrosomes, which is a first key insight into its mechanistic action. We agree that a deeper mechanistic understanding is always better, but even after 10 years of research on human MCAK, it is not quite clear how it contributes to spindle bipolarization in human cells: van Heesbeen et al shows that MCAK prevents a pushing force from astral MTs, but at the same time finds that this force is only a partial contributor. We therefore think a true understanding will take time, but that our work provides an important first step.

We respectfully disagree with the reviewer that our manuscript is built on two separate stories. Using the klp-7 mutant background, we identify PLK1 as a key regulator of LIN-5 in the context of centrosome separation, and we demonstrate that PLK1 also controls LIN-5 localization in a wild-type background, showing that our findings are valid beyond the klp-7 background. Nevertheless, we agree with reviewer 1 that our PLK1 data at this stage do not explain the AB vs P1 asymmetry seen in wild-type cells and we have now made this point even more explicit in the discussion (p16, lines 360-365).

With regard to the polarity-dependent delay, the necessity of partial depletion of PLK-1 does limit the possible conclusions. It is notable that the AB-P1 delay and corresponding LIN-5 asymmetry remain intact in plk-1(RNAi) in both WT and klp-7 backgrounds, consistent with residual PLK-1 activity, but this could also reflect an underlying PLK-1-independent asymmetry, leaving open the degree to which PLK-1 is required in WT for the delay. More clear is the effect of symmetric PLK-1 on the loss of the AB-P1 difference. It would be nice to confirm in their experiments that T186-
MEX-5 plus mex-6(RNAi) reproduces the delay and symmetric LIN-5 localization to eliminate roles for other upstream cues. This would be relatively convincing in terms of how the delay is achieved, but does not really address whether the observed delays are important to the embryo.

We agree with reviewer 1 that the fact that Plk1 depletion does not abolish the centrosome separation difference between AB and P1 cells in a wild-type and the klp-7 background, points to additional factor imposing this asymmetry, most likely via LIN-5. We now make this point more explicit in the discussion (p16, lines 360-365).

At the same time, we emphasize that uncovering a role of a protein via a sensitized background is not uncommon. Depletion of the human kinesin-12 Kif15 only affects centrosome separation when Eg5 function is partially impaired (Vanneste et al., Curr Biol., 2009; Tanenbaum et al., Curr Biol, 2009), yet it is recognized as a regulator of human centrosome separation.

The new temporal data is a good addition. However, it reveals a rather curious relationship between timing of separation onset and separation speed. AB separates later, but faster than P1. Similarly, klp-7 separates later, but faster than WT. Rather surprisingly, the postulated increase in pulling forces (plk-1) seems to change timing, but not speed of separation. Is it reasonable to expect that overcoming the intracentrosomal connections would depend on the magnitude of cortical pulling forces but the velocity of separation does not? Again, there is interesting data here, but it is not really discussed or explored further. I find it these results difficult to reconcile with the proposed model.

We thank reviewer 1 as his/her concerns raises two important points:

First, while our representation of the average centrosome-distance is a good read-out for the timing of centrosome separation it does not allow to cleanly extract centrosome separation speeds. Indeed, in some P1 cells the two centrosomes are already well separated (more than 8 microns) long before mitosis. This results in a high average inter-centrosome distance long before NEBD, but also an average centrosome separation speed that looks slower, since in those cells the two centrosomes cannot separate further. To avoid a mis-understanding, we now state this explicitly in the results (page 9, lines 166-173), and show examples of single cell trajectories in Supplementary Figure 1G.

Second, to test whether centrosome separation velocities themselves are different in AB and P1 cells and whether they are affected by KLP-7 depletion, we now quantify those values at the single cell level, taking in consideration the interval when inter-centrosome distances increase from 4 to 11 microns (or NEBD in the case of klp-7 AB cells). This analysis, shown in Supplementary Figure 1G and H, indicates that in contrast to centrosome separation timing, centrosome separation velocities are not significantly different in AB and P1 cells and that KLP-7 depletion does not affect those speeds either. Based on these data we indeed conclude that the cortical forces are rate-limiting for overcoming the inter-centrosomal connections, but not for the velocity of the separation itself. We now explicitly raise this point in the discussion, and speculate that other forces such as the centrosome to nucleus interaction or cross-linking elements on inter-polar microtubules might be rate-limiting for centrosome separation velocity (p17, lines 395-398).

Minor comments:

Line 166 - "KLP-7 specifically delays the initiation of centrosome separation in AB." I would argue that klp-7 delays both, albeit AB moreso, but also accelerates separation speed such that P1 largely catches up by NEBD

See comment above on centrosome separation

Western blots - While AB vs P1 westerns are not possible, par-2 vs par-3 would be relevant here. I don't feel this is necessary to address, but would confirm their conclusions versus prior observations.
Yes, this could be tested; however, since it is not possible to synchronize embryos, one will generate extracts from embryos at different stages, and most likely with cell of different fates, which would render any interpretation very difficult. We don’t think that such an experiment would be conclusive.