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Evidence for anaphase pulling forces during C. elegans meiosis

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Re: JCB manuscript #202005179

Prof. Francis J McNally
University of California, Davis
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Dear Prof. McNally,

Thank you for submitting your manuscript entitled "KNL-1 and KNL-3 are required for anaphase A pulling forces during C. elegans female meiosis". Thank you for your patience with the review process. Your manuscript has been assessed by expert reviewers, whose comments are appended below. Although the reviewers express potential interest in this work, significant concerns unfortunately preclude publication of the current version of the manuscript in JCB.

You will see that the reviewers provide lengthy but constructive and detailed assessments of the work. All three reviewers clearly appreciate the potential significance of this work. We have discussed the reviews editorially and feel that the reviewers raise important and valid points, including significant concerns about the data interpretation. We would be interested in considering a revised manuscript if you are willing to make essential changes to the manuscript as outlined by Reviewers #2 and #3 especially (their points #1 and #2) and below. There are two critical aspects:

1. While the demonstration of bivalent stretching supports the idea of pulling forces, these data are not a proof of end-on attachment mediated by KNL. Indeed, limited resolution of LM makes it impossible to definitively rule out contributions from lateral interactions. This should be acknowledged and the results should be interpreted in the context of available structural data. All three reviewers have excellent suggestions on how the text can be improved.

2. The issue of whether bivalents fail to separate in KNL-1/3 knockdown due to the absence of pulling forces vs. persistent cohesion needs to be addressed experimentally.

On the other hand, in our view, experimental additions suggested in Reviewer #1's point #6 (more phenotypic studies of spindle biology) are not necessary for the core conclusions to stand. While the issue of multipolarity in KNL-1/3 spindles is important, additional characterization of prometaphase with polar markers will not significantly improve the take-home conclusion of this work. The nature of microtubule interactions is likely to be the same in bipolar vs. multipolar spindles and pre-anaphase compaction of the spindle can be reliably detected even if the poles are not marked. Of course, it is up to you to decide how to respond to this point in your response to the reviewers' comments, but we editorially would not require additional experiments to address this point for publication.

Many of the Reviewers' concerns (Rev#1 in particular) will be addressed by a more complete description of methods and experimental setups. Providing details of image acquisition parameters is essential and needed for publication, as per the longstanding policy of the journal.

Lastly, I (Alexey) wanted to add that the notion that poleward pulling of chromosomes by the distal ends of short K-fibers is a part of normal anaphase (p.21, lines 15-18) has been explicitly demonstrated by Sikirzhytski and co-workers (same issue of the JCB as Elting et al., 2014).

Please let us know if you are able to address the major issues outlined above and wish to submit a revised manuscript to JCB. Note that a substantial amount of additional experimental data likely would be needed to satisfactorily address the concerns of the reviewers. We would be happy to further discuss the revisions if you have any questions or anticipate any issues addressing the reviewers' remarks.

As you may know, the typical timeframe for revisions is three to four months. However, we at JCB realize that the implementation of social distancing and shelter in place measures that limit spread of COVID-19 also pose challenges to scientific researchers. Lab closures especially are preventing scientists from conducting experiments to further their research. Therefore, JCB has waived the revision time limit. We recommend that you reach out to the editors once your lab has reopened to decide on an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

If you choose to revise and resubmit your manuscript, please also attend to the following editorial points. Please direct any editorial questions to the journal office.

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Text limits: Character count 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: Your manuscript may have up to 10 main text figures. To avoid delays in production, figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, http://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

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Supplemental information: There are strict limits on the allowable amount of supplemental data. Your manuscript may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

If you choose to resubmit, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

Regardless of how you choose to proceed, we hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised. You can contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for thinking of JCB as an appropriate place to publish your work.

Sincerely,

Alexey Khodjakov, PhD
Monitoring Editor, Journal of Cell Biology

Melina Casadio, PhD
Senior Scientific Editor, Journal of Cell Biology

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

Danlasky et al describe their analysis of the requirements for outer kinetochore proteins during C. elegans oocyte meiotic cell division. This has been an exceptionally controversial topic, with published work arguing that kinetochores are not required for anaphase/chromosome segregation during C. elegans oocyte meiotic cell division, that lateral attachments rather than end-on attachments of microtubules to chromosomes are responsible for generating chromosome movement, and that anaphase B is driven entirely by central spindle pushing forces without a role for kinetochores. Most notably, a previous influential study by Dumont et al 2010 Nature Cell Biology (cited extensively in this manuscript) provided evidence that while outer kinetochore proteins are required for congression of chromosomes to the metaphase plate, they dissociate from chromosomes during anaphase are not required for anaphase chromosome movements.

Danlasky et al confirm the congression defects first described by Dumont et al, and they nicely quantify the congression defects in Figure 3. But Danlasky et al now argue that in fact kinetochore proteins are required for pulling forces on chromosomes that (i) promote kinetochore stretching early in meiosis I, with the presumably associated pulling forces possibly accounting for the congression defects; (ii) generate anaphase A chromosome movement toward the spindle poles during meiosis I, and (iii) are required to separate the homologs in bivalents during meiosis I anaphase. They also show that outer kinetochore proteins (KNL-1 and the MIS-12 complex component KNL-3) only partially dissociate from kinetochores during anaphase, with substantial levels remaining associated with kinetochores throughout anaphase during oocyte meiosis I, transitioning from kinetochore cup structures to ring structures that thus provide surfaces for possible attachment to microtubules both from the spindle poles and from the central spindle during anaphase chromosome movements. The authors provide the most impressive and highest resolution light microscopy imaging yet published of kinetochore protein localization and spindle structure during oocyte meiosis I in C. elegans (using in part a strain they previously generated with CRISPR to fuse GFP to both KNL-1 and KNL-3). Motivated by the Dumont et al observation that KNL-3 kinetochore localization does not depend on KNL-1, they also use CRISPR to degron tag both KNL-1 and -3 in the same strain, and knock down KNL-1 and -3 function simultaneously (both with auxin treatment for the degron tagged strain, and GFP RNAi for the strain in which both are tagged with GFP; these give nearly identical phenotypes). These improvements in methodology have led to substantial new insights that greatly advance our understanding of the requirements for outer kinetochore proteins during C. elegans oocyte meiosis, and provide clear evidence that they have far more extensive requirements than has been previously appreciated. Perhaps the most compelling finding is that most homologs fail to separate after KNL-1/3 knockdown, with intact bivalents typically moving toward one pole (some bivalents moving to one pole and some to the

other). Thus the anaphase movements that have been documented previously were in fact due to an extremely abnormal anaphase (without homolog separation), and/or perhaps also to less complete knockdown of kinetochore function (with RNAi targeting only KNL-1 or KNL-3 or other individual outer kinetochore components in previous studies). These findings provide a major correction to our understanding of the requirements for outer kinetochore proteins during oocyte meiosis I in C. elegans.

Danlasky et al also argue that highly redundant factors mediate microtubule/chromosome attachments to contribute to anaphase movements during oocyte meiosis I, based almost entirely on negative results involving the simultaneous knockdown of both KNL-1 and -3 and other oocyte chromosome or spindle associated proteins.

While Danlasky et al provide important new insights into the roles of kinetochore proteins (specifically KNL-1 and -3) during C. elegans oocyte meiotic cell division, the manuscript suffers from numerous problems that require extensive clarification before the manuscript can be considered suitable for publication in The Journal of Cell Biology. Indeed, the manuscript could possibly be greatly improved by eliminating much of the peripheral data and figures and focusing more narrowly on the positive results involving requirements for KLN-1 and -3. However, even these requirements require substantial clarification for readers to be able to critically evaluate the validity of the authors' conclusions concerning the requirements for KNL-1 and -3, and the manuscript fails to adequately describe the phenotype that results from the simultaneous knockdown of both KNL-1 and -3.

Major comments.

1. The authors do not adequately describe how their live imaging data were collected. In the Methods, they refer to 5, 10 or 15 second intervals but rarely specify what time intervals were used in the different figures. More importantly, they never state how many focal planes were collected in the z-axis for any of the figures, or how many of the focal planes were used for the images shown in the figures. They often do not state in the legends or elsewhere if maximum projection images are shown, or if the images were processed in other ways. The authors need to provide more detail on the imaging throughout the manuscript.

2. There are numerous times in the manuscript when the authors do not provide adequate information on the time points used for their quantifications. Without more such information, it is impossible to critically evaluate the validity of their conclusions, or the objectivity of their analyses. Examples of this include. The first examples apply to Figure 1 and in part their description on page 6, lines 3-7: the authors refer to metaphase and the "ring stage of anaphase" when describing the decrease in KNL-1/3 levels over time. What time points were used in the examples shown in Figures 1A? What time points were used for the calculation of the mean values? The "ring stage of anaphase" is especially vague, as the authors point out that one can detect rings over a substantial span of time during anaphase B. It also would be helpful if the authors could note the time points shown in Figures 1B and 1D.

3. In Figure 1, panels D-G seem largely peripheral to the roles of KNL-1 and -3 and the text describing them is very confusing. On page 7, lines 1-9, the authors repeatedly refer to various proteins or chromosomes occluding other proteins. What do the authors mean by occlusion? What do they mean by "spindle pole material?" What seems clear is that the rings form independently of the "linear elements" and independently of ROD-1 and ASPM-1. But what does occlusion mean or have to do with any of this? Proteins can diffuse and re-bind anywhere in a cell, and it is not at all clear how one protein (or chromosome) in one place might occlude another protein from being there

(as far as I know, occlude just means block). And what kind of "optical artifact" are the authors referring to in line 9? This entire section and panels 1D to 1G seem tangential to the main points of the paper and the corresponding text is incomprehensible to this reviewer. These panels could easily be eliminated and the focus of the paper narrowed to more thoroughly and clearly explain the more important data and points. Also, in line 11, the authors refer to the time points 7:30 - 9:50 in Figure 2A, but the last time point in Figure 2A is 5:30.

4. The conclusion that KNL-1/3 are required for kinetochore stretching (Figure 4) cannot be critically evaluated without more information as to how the authors made their measurements.

First, how do the authors define metaphase for KNL-1/3 when their own data shown that chromosomes never align to form a metaphase plate? The Methods simply say that metaphase measurements were taken before spindle shortening began. How long before spindle shortening began (and also see major comment #5 below about spindle morphology)? The pre-anaphase measurements (presumably this should be called pre-anaphase A to be more clear) are said to be made between 90-120 seconds before homolog separation, with line scans used to assess separation. While this sounds reasonable, it would help to know how the authors chose 90 vs 100 vs 110 vs 120 for their time points.

Second, the authors measure two distances: homolog center to center, and outer kinetochore edge to outer kinetochore edge. The schematics show the homologs as nice perfect spheres, but in the images shown in Figure 4A the homologs are highly irregular in shape. How did the authors impose such perfect spheres onto these highly irregular shapes? How did the authors define the "edge" of the outer kinetochore cups?

Third, the authors never refer to how the spindles might not be aligned along the plane that is parallel to the cover slips in their preparations. If the spindles are not parallel to the planes of the z-stacks, then the authors would need to use 3-D imaging and rotations to accurately measure the distances. The authors need to explain how they measured distance given the issue of spindle alignment relative to the plane of imaging.

The differences in these length measurements are very modest (though significant as shown), but without more information (and perhaps supplemental figures showing the examples used) it is impossible to judge the validity of the measurements and the differences. This is a key conclusion for the authors but the validity and objectivity of the measurements cannot be assessed with the information given.

5. One last concern about the stretching data: the inference that the lack of stretching (if that is a valid conclusion) might explain the congression defects does not seem reasonable, given that the authors are measuring a process that occurs in control oocytes well after congression is complete

6. The authors do not adequately address the consequences of KNL-1/3 knockdown on spindle morphology, and in particular how normally or abnormally the mutant oocytes do or do not establish spindle bipolarity. The manuscript could possibly be much improved with more document of spindle bipolarity, using the GFP::ASPM-1 pole marker. The images shown in Figure 3A indicate some degree of spindle disorganization; in particular the middle example of the auxin treatment is highly irregular and not obviously bipolar. A single image of a partially bipolar spindle is shown using GFP:::LIN-5 as a pole marker. And in Figure 6 (panels B-E), it appears that nearly half of the spindles are abnormal in being "3-way" or "4-way", which seems to indicate tripolar or tetrapolar structures. Given that the authors rely on defining metaphase as being "before spindle shortening begins", the

authors should more thoroughly document the impact of KNL-1/3 knockdown on spindle morphology over time, and document exactly how they can pinpoint when spindle shortening begins. Such information is critical to evaluating the validity of the measurements on kinetochore stretching and anaphase velocities. The manuscript would greatly benefit from more attention to this issue, rather than focusing on the curious business of occlusion and optical artifacts in Figure 1, the questionable/peripheral value of the him-8 analysis in Figure 7, and all of the largely negative (and due to unknown levels of functional depletion using RNAi/AID, entirely inconclusive) data on "highly redundant" factors contributing to chromosome movements/attachments in Figures 8-10 that is very much peripheral to the key points of the manuscript.

7. The data on KNL-1/3 being required for anaphase A but not anaphase B (Figure 5) is compelling but again the authors do not provide adequate information for critical evaluation of their conclusions, as to the time points being used for these velocity measurements. In panels 5G, 5G and 5I, the authors show data from representative oocytes. How did the authors define t = 0? What is the relevance of the double arrows to the velocity measurements? The legend says they "indicate the initiation of anaphase chromosome movement relative to spindle shortening/elongation cycle." I have no idea what that sentence actually means. Does it refer to anaphase A or B? To when spindles begin to shorten, or begin to lengthen, or what? And what do those double arrows have to do with how they actually measured velocity for either A or B? It would be much more helpful if the authors could simply indicate with arrows and arrowheads what time interval was used for A and what interval for B in those examples. Maybe similar plots could be shown in supplemental data for the other oocytes. The legend says that anaphase A was measured over the 80 seconds before the beginning of spindle elongation, so that beginning could easily be shown with an arrowhead. Similarly, for anaphase B velocity (Figure 6A), the authors could indicate what interval constitutes "spindle elongation".

8. Another clarification on anaphase A and B movements concerns the authors documentation of "pop-out" chromosomes (Supplemental Figure 2C), also described as 3-way and 4-way spindles in Figure 6C and 6C. When the authors measured anaphase B velocities, did they only use bivalents that were not at the spindle edges, o pop-out, or 3-way, or 4-way? The bar graph in Supplemental Figure 2C indicates that about 50 of the bivalents were "pop-out". How did the authors judge if a spindle had a "pop-out" structure and which spindles were used to assess anaphase velocities?

9. One final clarification on anaphase chromosome movements. It is very intriguing that intact bivalents move together to a single pole after KNL-1/3 knockdown. But the authors do not devote any discussion to how this movement is being promoted. Indeed in Figure 5C, there appears to be very little detectable tubulin inbetween the segregating pair of bivalents. The authors should provide some discussion as to how they think these anaphase B movements are being promoted.

10. The authors conclude that bivalents remain intact after KNL-1/3 knockdown due to a lack of pole-ward pulling forces early in anaphase. To support this interpretation, they show that both AIR-2/Aurora B and Separase show roughly normal dynamics in their localization after KNL-1/3 knockdown. An alternative interpretation is the KNL-1/3 are in fact required for cohesin cleavage, even though AIR-2 and Separase are present in roughly normal locations. Indeed, it is striking how normal the bivalents appear even after moving substantial distances toward a pole. One might just as easily argue that the homologs remain paired due to a failure to cleave cohesins and that KNL-1/3 are somehow required for this processing. The authors should at least acknowledge this alternative view and perhaps discuss how the bivalents could remain so normal looking in their morphology if cohesins have been cleaved. One additional piece of information that might help in evaluating this alternative explanation would be to know if the homologs and sister chromatids do

come apart during anaphase of meiosis 2.

11. The data in Figures 8-10 and Supplemental Figures 4 and 5 seem very peripheral to the main points of the paper and while they involve some impressive genetic manipulations do not really contribute to understanding the roles of KNL-1 and -3. Moreover, the conclusions are very tentative, given that the depletions of the various proteins by RNAi or AID may well be incomplete with significant function remaining, a caveat the authors never even once acknowledge. Moreover, the authors devote an entire supplemental figure to KLP-7 localization and yet never knock down its function in the KNL-1/3 kd background. It seems the authors could drop these figures and publish them elsewhere, as they more distracting than helpful in this context, and the authors would be better served by address some of the clarifications that are needed and noted in the other major comments.

Minor Comments.

1. The authors note in the Introduction that knockdown of KNL-3 rescues anaphase in mutants lacking MEL-28 (Hattersley et al.., 2016). This is an intriguing observation that argue against a requirement for KNL-3 in anaphase forces, and the authors never return to how they might explain this observation in light of their findings and conclusions about KNL-1 and -3 requirements. Indeed, the Discussion in general does not address very much of their own data and its relevance to the controversy over kinetochore function in C. elegans, but rather focuses extensively on spindle cutting studies and on the issue of redundancy of microtubule attachment to chromosome mechanisms, whereas the focus of the most important data in this paper is on KNL-1/3 function.

2. The Discussion also could be improved by including some reference to what is known about microtubule/chromosome attachments to chromosomes during oocyte meiosis in other organisms. The generality of the authors' findings with respect to oocyte meiotic cell division in other model systems is almost completely ignored.

3. Page 7, line 3: the authors refer to chromosomes being "embedded" in spindle pole material. What material are they referring to? Is it embedded or simply adjacent to it?

4. In Figure 2B, the authors might want to provide small arrowheads pointing to the fingers of KNL-1/3 projecting toward the poles.

5. Page 10, line 4: the authors refer to Figure 4A-E; there is no E

6. Figure 5G-I: would it be possible to combine the data from all oocytes into one graph for each genotype, and show standard deviations? Or are the time courses highly variable from oocyte to oocyte? Again, more information on the spindle morphology over time, and the timing of events during meiosis I after KNL-1/3 knockdown might clarify some of the conclusions and arguments.

7. The authors state that KNL-1/3 might be involved in the stretching of the X chromosomes in him-8 mutants. What happens if you knock down KNL-1/3 in the him-8 mutant background?

8. Page 14, lines 1-2: the authors should define "-1 oocyte" for a general audience

9. Page 21, line 4: presumably the authors mean "non-NDC-80-dependent pulling forces persist..." and should make this more clear. However, again the authors fail to mention the caveat that their RNAi might not eliminate NDC-80 function and therefore they cannot conclude that other factors

contribute to pulling forces.

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

The manuscript by Danlasky et at examines the forces driving chromosome poleward movement during anaphase in C. elegans meiosis. Experiments in many cell types reveal poleward segregation relies on kinetochore/microtubule dependent pulling forces. In contrast for C elegans meiosis, it has been proposed that anaphase A is driven by a kinetochore-independent microtubule-pushing mechanism involving microtubule associations with the inner chromosome face. However through a combination of cellular and functional genetic analysis, Danlasky and colleagues challenge this view. They find that the core kinetochore proteins KNL1 and KNL3 are required for anaphase stretching suggesting a role for these proteins in pulling rather than pushing. This conclusion is supported with fluorescent analysis of KNL localization. Overall the manuscript is well-written and the quality of the data is excellent. However there are a number of major and minor issues regarding the interpretation of the results and conclusions that must be addressed in order for the manuscript to be acceptable for publication:

Major:

1) The evidence for a conventional end-on "pulling" force driving chromosome separation is not compelling. The KNL-1,3 cups that are proposed to mediate this pulling force via potential end-on attachments also extend laterally around the chromosomes (there is also a high concentration of MTs that seem to be associating laterally here as well). Thus the defects observed upon KNL-1,3 knockdown could be due to diminished lateral interactions as opposed to end-on interactions. This isn't to say that there isn't end-on pulling, but the language used is fairly strong and doesn't seem to take into account these potential lateral interactions. I also think additional discussion is needed to reconcile the interpretation of a pulling force with the fact that severing MTs on the poleward side of chromosomes doesn't stop poleward movement in C. elegans. I know there is some discussion here, but the discussion really doesn't seem to address what is going on in anaphase C. elegans oocytes in particular, which appear to be a special case (as opposed to metaphase PtK2 cells).

2) Stronger evidence is needed to demonstrate that the congression defect is truly unlinked from the separation defect. The mei-2 mutant is a good idea, but the congression defect does not seem to be as severe as the knl-1,3(kd) congression defect (i.e. no "stacked" bivalents). Along those lines, the mei-2 mutant does have some separation defects as well. So could the mei-2 be an intermediate between both? A more thorough characterization of the mei-2 congression defects (directly comparing to the knl-1,3(kd) congression defects would strengthen the authors' claims.

Other and related issues to be addressed:

Figure 1
-Could the GFP affect stability/protein turnover rate? (A-B)
-Stain endogenous KNL-1/3 (not GFP-tagged) still see same "ring structure" in anaphase B?
-Midzone microtubules would have - ends contacting segregating chromosomes? (C)
-Why does Figure 1C look different than Figure 1F? - and seemingly discussed differently in the results as well (1C: ASPM-1 is surrounding KNL 1/3 rings (which in turn surround chromosomes); 1F: chromosomes are not surrounded by ASPM-1 due to spindle pole material)

Figure 2
-Figure 2A does not have timepoints 7:30-9:50 as indicated in the text (pg 7, line 11)

Figure 4
-When is the timepoint for "metaphase" considered?
-How many timepoints before initiation of chromosome movement is "pre-anaphase"?
-A good control would be to show that the distance between the midzone-facing sides of homologs does not change between "metaphase" and "pre-anaphase"

Figure 5
-Congression defect in mei-2 mutant doesn't look as severe as that of the KNL 1/3 knockdown (i.e. chromosomes look closer to the metaphase plate and there doesn't appear to be the bivalent stacking as in the KNL-1/3 knockdown). Could a similar analysis be done as in Figure 3C for the mei-2 mutant? If the distribution is similar between the two mutants, it would strengthen the interpretation from this experiment.
-as it is now, it looks like the mei-2 mutant has both a milder congression defect and a milder homolog separation defect
-Tying into the point above, does separation defect only occur when bivalents are stacked (as in Fig 5B, C)?
-For 5H, instead of (or in addition to) measuring the distance between between intact bivalents, could the distance be measured between segregating homologs in the population of homologs that do separate (~25?). Would they show anaphase A movement?

Figure 8
-In text reference to 8D: "metaphase congression defects were observed in a significantly lower fraction of NDC-80-depleted embryos than in knl-1.3(kd) embryos" and gives some data, but the figure panel 8D doesn't contain any KNL-1,3(kd) data was this from the same experiment in 3C?

Figure 9
-Parts of 9E-F are missing "n" values (blue control in E also has a discrepancy in number of points (9) and "N" value (10))

Figure S3
-Panel C, in texts states that the width difference between control metaphase and control pre-anaphase is significant, but this is not illustrated in the figure panel
-Panel D, what does the blue dot mean?
-Panel E, in text is highlighting no difference between metaphase v pre-anaphase, but in the panel is highlighting no difference between control and knl 1,3 kd area

Figure S1
-Would be helpful to see the whole cell at metaphase in addition to the panels showing only one bivalent (Panels A-D)
-E-F, separase localization looks different between control and RNAi images (control: separase localization is perpendicular to division axis; RNAi: separase localization is parallel to division axis) why? Is this difference representative?

Figure S4
-Panel B, in text suggest that AIR-2 dissociates from chromosomes in anaphase similarly between knl knockdowns and controls, but it looks like there is still AIR-2 localization on chromosomes in

anaphase B (5:10 panel)

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

This manuscript by Danlasky et.al. addresses the role of kinetochores during C. elegans female meiosis. This is an important area of investigation because previous work by others suggested that chromosome segregation in this system is kinetochore-independent. The authors demonstrate that following kinetochore depletion (via degron-mediated depletion of KNL-1 and KNL-3), chromosomes fail to properly align and orient on spindles, consistent with previous work. Moreover, they also go on to demonstrate that there are severe segregation defects in anaphase following kinetochore depletion. These authors previously showed that chromosomes stretch at the metaphase-to-anaphase transition, suggesting the existence of pulling forces at that stage, and in the current manuscript they demonstrate that this pulling is kinetochore-dependent. Interestingly, the authors also demonstrate that homologs fail to come apart in kinetochore-depleted anaphase, and instead move to spindle poles as intact bivalents. These findings demonstrate that, in contrast to previous studies, kinetochores do play important roles in chromosome segregation in C. elegans oocytes.

This manuscript reports important findings that have the potential to significantly advance thinking in this field. However, as detailed below, in some cases the data in the manuscript is not strong enough to support particular conclusions. I would like to emphasize that the extensive nature of the comments below is my attempt to improve a manuscript that I think could make an important contribution to the field; please do not interpret the length of my comments as an attempt to reject it outright.

Major points

1. The evidence that bivalents stretch at the metaphase to anaphase transition, and that this stretching is dependent on KNL-1/3, is strong. This data nicely supports the idea that there could be end-on pulling at this stage to mediate anaphase A, as the authors suggest. However, in other parts of the manuscript, the authors suggest that end-on attachments exist at other stages, but these claims are not well supported in light of previous work in the field; if the authors want to make these claims and refute previous studies, their data needs to be stronger and more convincing. Figure 2 in particular seems to be used to suggest that there could be end-on kinetochore attachments in metaphase, but this is not convincing. Low resolution live imaging cannot provide information about whether there are end-on attachments to chromosomes. In Redemann 2018, they specifically look for microtubule ends associating with the ends of bivalents, within 250nm of the chromosome surface (this is the ribosome-free zone that has been defined as the kinetochore in EM images). In metaphase, they found very few microtubules in this zone (the ones they found looked to be predominantly laterally-associated, and they did not find evidence for end-on attachments (Figure 1C, S1 of that paper). Redemann also looked at mid-late anaphase and also did not see a major population of microtubules on the outside surfaces of chromosomes (Fig 1D, 1E, S2). I don't think that the data presented in Figure 2 is strong enough evidence to refute this published data and to suggest that there may be end-on pulling interactions in metaphase. However, I do think that it is possible that upon spindle shrinkage at anaphase onset, transient end-on kinetochore attachments may form that could exert pulling force. This is better supported by the authors' data because they see chromosome stretching/narrowing at that stage (which nicely supports the idea of "pulling" at that specific stage) and also because that stage has not been

described by EM - the spindles published are either in earlier anaphase, or at the end of anaphase A, when these transient end-on attachments could have disassembled. I think that the data in this manuscript is consistent with a lack of end-on attachments prior to anaphase, the establishment of end-on attachments at the metaphase-to-anaphase transition to mediate the stretching/pulling of chromosomes (and anaphase A), and then a switch to anaphase-B spindle elongation as the major form of segregation. This view better aligns with other published work in the field.
Since I don't think that this manuscript provides strong evidence for end-on attachments prior to the metaphase-to-anaphase transition, I think that the authors should reconsider the conclusions drawn from Figure 2, and revise strong statements proposing pulling forces prior to anaphase onset throughout the manuscript. As an example, the authors propose that the congression defects following KNL-1/3 depletion are due to lack of poleward pulling forces on chromosomes; this statement should be removed or softened since these proposed prometaphase pulling forces would presumably be from end-on attachments, which the data in the paper does not convincingly demonstrate are present.
I also suggest that the authors consider and discuss the ideas proposed in Davis-Roca 2017, which hypothesized that there could be a transient acquisition of end-on attachments to mediate chromosome stretching/anaphase A. That paper proposed that the pulling forces were transient, and under normal conditions (when the majority of kinetochore signal dissociates from chromosomes), these end-on attachments would not persist. However, they also found conditions where kinetochore proteins remained brightly on chromosomes, and suggested that under those conditions the end-on attachments/pulling forces could remain. I encourage the authors of the current manuscript to consider whether the findings of Davis-Roca 2017 could fit into their thinking/model. Is it possible that any of the conditions examined (e.g. him-8 mutant with univalents) are conditions where kinetochore proteins are retained more strongly on chromosomes, to keep strong end-on attachments that mediate pulling forces (whereas usually these pulling forces would dissipate when the levels of kinetochore proteins are decreased)? This interpretation would fit better with the Redemann EM data that did not find evidence for end-on attachments in mid-late anaphase under normal conditions.

2. The evidence presented that KNL-1/3 depletion blocks separation of homologs in anaphase is very strong. However, the interpretation of this result by the authors (that homologous chromosomes need to be pulled apart by KNL-1/3 dependent forces, otherwise they behave as a single intact unit) is difficult to reconcile with previous studies in the field. If I understand the model presented in this paper correctly, bipolar pulling forces mediated by end-on attachments to kinetochores are required to pull chromosomes apart in anaphase A, and this pulling is required for bivalents to come apart in anaphase. However, there are many other mutant conditions where there are not bipolar pulling forces in anaphase A, and chromosomes are still able to separate from each other. For example, chromosomes still come apart during anaphase on monopolar spindles (they move to the same pole, but not as an intact unit), and chromosomes also segregate following depletion of KLP-15/16; in both of these mutant conditions there is no bipolar spindle at anaphase onset to exert Anaphase-A-like bipolar pulling forces. In my mind, this makes it more likely that KNL-1/3 depletion is affecting the actual physical separation of chromosomes (e.g. by affecting separase cleavage of cohesin), instead of the interpretation presented in the paper (that bipolar pulling forces are required for individual chromosomes with cleaved cohesin to move to opposite poles). Although the authors show separase staining following KNL-1/3 depletion in Figure S1F, the anaphase picture is very messy, and does not show early anaphase (the chromosomes have already moved far apart), so this data does not clearly demonstrate that separase localizes normally to the midbivalent at the stage when it would be expected to cleave cohesin; providing more convincing evidence that separase localizes normally at anaphase onset is essential if the authors want to make this claim. However, even if the authors could provide this evidence that separase localized

normally, this would not serve as proof that cohesin was properly cleaved, so this should be taken into account in any re-writing of the narrative.
Therefore, I would encourage the authors to consider and discuss alternate hypotheses that could explain their results, and to discuss their result in the context of the other previous findings in the field. Also, for this manuscript, it is important for the authors to demonstrate whether chromosomes EVER separate from one another in KNL-1/3 depletion, by analyzing MII. This could shed light on whether homologs dissociate from one another (via cohesin cleavage) but move together to the same pole (i.e. they separate, but kinetochore pulling is required to get them moving in opposite directions), or whether homologs do not come apart (Movie S5 progresses to MII, but it was hard for me to determine if the bivalents ever dissociated into individual chromosomes). The latter result would be more consistent with a model in which KNL-1/3 depletion alters chromosome structure in a way that prevents homolog dissociation. The authors show in Figure 10 that KNL-1/3 depletion removes the midbivalent population of KLP-7; this suggests that there are some changes in the middle region of the bivalent following depletion of kinetochore proteins, which could also affect homolog dissociation. This possibility should therefore be discussed.

Other points:

- Page 6 lines 14-15: The statement that ASPM-1 surrounds the kinetochore rings is not clear from the data presented in Figure 1C. ASPM-1 appears enriched on the outside of the chromosomes, but if there is an enrichment on the inside surface of chromosomes it is faint and very hard to see. If the authors want to make this claim they should present more convincing data.
- The authors talk about the fact that kinetochore rings could provide a means of attachment to the inside surfaces of separating chromosomes (page 6 lines 12-14; page 7 lines 9-10; page 22 lines 8-11). However, it is my understanding that in the Laband/Dumont model, the ends of microtubules that are pushing on the inside surfaces of chromosomes would be predicted to be minus ends, not plus ends (plus end polymerization in the center of the spindle would provide force for the minus ends to push on the chromosomes). Therefore, the suggestion that the kinetochore rings could provide a means of end-on attachment to microtubules on the inside surfaces of chromosomes is confusing to me. Is there any evidence that kinetochores can make end-on attachments to microtubule minus ends?
- I did not understand the sentence on page 7 lines 10-11 that cites Fig. 2A ("as further suggested by…). The timestamps noted in the figure callout do not exist in the figure (which only goes until 5:30). Additionally, even if the timestamps were just typos and this callout is referring to some of the frames shown in Fig. 2A, these images are not at high enough resolution to draw conclusions about whether there are microtubule attachment points on the inside surfaces of chromosomes.
- Figure 2: Details of the quantification are not in the materials and methods. Are the images in Figure 2A and 2B max projections or single slices? Was the quantification done on single slices?
- Page 9 lines 3-5 states that the bivalents on the outside of the spindle have "no apparent microtubule contacts on one or more sides of the bivalent", but the spindles in Figure 3A and B that are referenced have bivalents on the outside of the spindle appear to have contacts on the sides. I might be reading too much into the wording of the sentence, but do you mean that they don't have contacts on the microtubule ends? It would also be helpful to put arrows or arrowheads pointing to the bivalents you are referring to, to help the reader.
- Page 9 line 4 has a figure callout to Fig. 4 A-E, but there is no Fig. 4E
- Page 9: It would be helpful to define "inter-homolog" and "intra-homolog" stretch the first time you use them, as these terms are not intuitive to the non-expert reader
- Page 9 lines 9-13: the authors should remove or soften the suggestions that the microtubule channels are caused by exclusion of microtubules from bivalents. The authors use the data in Figure 2 to make this point (which, as discussed in major point #1, I do not find convincing).

Moreover, they also claim that the channels could be gone following KNL-1/3 depletion because the bivalents would be smaller (since they lack proteins cupping the ends). However, Figure 4A shows that there are still cups of some proteins on bivalents following KNL-1/3 depletion, so it is not clear how much smaller the bivalents would be.
- Page 12 line 5: The sentence talks about fibers pushing on the inner faces of chromosomes, but in the case of KNL-1/3 depletion, the inner face is not exposed (since the bivalent is intact), so it would be the outer surface. Rephrase sentence to avoid confusion.
- Page 12 line 16: The phrase "pushing between chromosomes" is confusing, because it suggests chromosomes have separated. I think rephrasing to "pushing between intact bivalents" might prevent confusion.
- Figure 7: Although it is clear that KNL-1/3 are on univalents as they stretch, the images in Figure 7 did not look like stretched KNL-1/3 rings - it simply looks like these proteins are coating the univalent. I therefore suggest changing the wording of the header on page 12 line 19 (and other references to stretched rings) so the reader is not confused. I don't think this affects the point you are trying to make (that kinetochore proteins are retained), but it will better reflect the data.
- Page 12 lines 8-11: The authors appear to imply that the KNL proteins on the univalent are physically coupled to the KNL rings on the other homologs, but the images are not at high enough resolution to state this. Just because there are kinetochore proteins surrounding chromosomes and the chromosomes are close together does not mean that they are physically coupled. Therefore, the statement that the rings are contiguous should be removed.
- Page 14 line 19: change "separation" to "velocity" since homologs don't separate in kinetochore-depleted spindles.
- Page 16 line 23: "...must require attachment of chromosomes to the ends of elongating microtubule bundles...". It is possible that chromosomes could also associate laterally with microtubules. Change to "...ends or sides..."
- Page 17 line 10: This line references the "AIR-2 ring", but the cited papers show that AIR-2 leaves the ring early and relocalizes to microtubules, while other components remain in the ring structure longer, so the reference to the "AIR-2 ring" is not accurate. Rephrase statement.
- Page 17 lines 11-14: The authors state that their live imaging shows that rings "remain attached to chromosomes" but this is not shown by the data. Just because the rings are near chromosomes is not evidence that they are attached (the images are not high enough resolution to show this). Also, in Figure S4C, chromosomes are not shown. Higher resolution imaging in the Davis-Roca papers suggests the rings are removed in early anaphase and can remain as intact units (see Davis-Roca 2018, Figure 2 as an example). The data in the current manuscript are not convincing enough to argue against the view that the rings dissociate from chromosomes at early anaphase.
- Page 18 lines 7-10: I don't understand this sentence connecting the NDC-80 depletion results to a conclusion about ring elongation. Please rephrase/explain better.
- Page 21 line 1: Given my concern in major point #1 above (that there is not convincing evidence that there are end-on attachments prior to spindle shrinkage), I would suggest rephrasing "...may increase during spindle shortening to mediate this increased pulling" to "...may be established during spindle shortening to mediate this pulling".
- Page 21 lines 2-4: I don't understand this sentence, please rephrase. How does the fact that chromosomes move to poles following NDC-80 depletion support the persistence of pulling forces?
- Figure 10: Put a label on Figure 10B so that it is clear what is being depleted in the plus auxin condition (same comment for Figure 5B). Since you use a couple of degron strains in the paper (KNL-1/3, dynein), it will help to label this in every figure for clarity.
- Figure S2: in the graphs in S2B and D, there are 2 "Ns" listed for each ("N" and "n"). State in the figure legend the difference between these numbers.
- Figure S4E: How was depletion of GEI-17 confirmed? Since it is labeled with GFP in a strain where the microtubules are also GFP-tagged, this is impossible to see in the images (and I didn't see any

other attempt to confirm depletion in other figures).

Typos:
- Figure 8 legend: intensty should be intensity
- Page 9 line 8: duplicate references to Vargas 2019
- Page 21 line 12: experments

Response to editor and reviewer comments

We thank the editors and reviewers for taking the time to provide us with these thoughtful comments.

You will see that the reviewers provide lengthy but constructive and detailed assessments of the work. All three reviewers clearly appreciate the potential significance of this work. We have discussed the reviews editorially and feel that the reviewers raise important and valid points, including significant concerns about the data interpretation. We would be interested in considering a revised manuscript if you are willing to make essential changes to the manuscript as outlined by Reviewers #2 and #3 especially (their points #1 and #2) and below. There are two critical aspects:

1. While the demonstration of bivalent stretching supports the idea of pulling forces, these data are not a proof of end-on attachment mediated by KNL. Indeed, limited resolution of LM makes it impossible to definitively rule out contributions from lateral interactions. This should be acknowledged and the results should be interpreted in the context of available structural data. All three reviewers have excellent suggestions on how the text can be improved.

We have added more extensive discussion of lateral vs end on attachment.

2. The issue of whether bivalents fail to separate in KNL-1/3 knockdown due to the absence of pulling forces vs. persistent cohesion needs to be addressed experimentally.

We have added a supplemental video and numerical data in the results text showing that most bivalents that end up at one pole during early anaphase split into two homologs by the end of anaphase I. We also state the possibility that KNL-1,3 are positive regulators of separase.

On the other hand, in our view, experimental additions suggested in Reviewer #1's point #6 (more phenotypic studies of spindle biology) are not necessary for the core conclusions to stand. While the issue of multipolarity in KNL-1/3 spindles is important, additional characterization of prometaphase with polar markers will not significantly improve the take-home conclusion of this work. The nature of microtubule interactions is likely to be the same in bipolar vs. multipolar spindles and pre-anaphase compaction of the spindle can be reliably detected even if the poles are not marked. Of course, it is up to you to decide how to respond to this point in your response to the reviewers' comments, but we editorially would not require additional experiments to address this point for publication.

Many of the Reviewers' concerns (Rev#1 in particular) will be addressed by a more complete description of methods and experimental setups. Providing details of image acquisition parameters is essential and needed for publication, as per the longstanding policy of the journal.

We have expanded the Materials and Methods to provide these details.

Lastly, I (Alexey) wanted to add that the notion that poleward pulling of chromosomes by the distal ends of short K-fibers is a part of normal anaphase (p.21, lines 15-18) has been explicitly demonstrated by Sikirzhytski and co-workers (same issue of the JCB as Elting et al., 2014).

We have added this reference and changed the text accordingly.

Please let us know if you are able to address the major issues outlined above and wish to submit a revised manuscript to JCB. Note that a substantial amount of additional experimental data likely would be needed to satisfactorily address the concerns of the reviewers. We would be happy to further discuss the revisions if you have any questions or anticipate any issues addressing the reviewers' remarks.

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

Danlasky et al describe their analysis of the requirements for outer kinetochore proteins during C. elegans oocyte meiotic cell division. This has been an exceptionally controversial topic, with published work arguing that kinetochores are not required for anaphase/chromosome segregation during C. elegans oocyte meiotic cell division, that lateral attachments rather than end-on attachments of microtubules to chromosomes are responsible for generating chromosome movement, and that anaphase B is driven entirely by central spindle pushing forces without a role for kinetochores. Most notably, a previous influential study by Dumont et al 2010 Nature Cell Biology (cited extensively in this manuscript) provided evidence that while outer kinetochore proteins are required for congression of chromosomes to the metaphase plate, they dissociate from chromosomes during anaphase are not required for anaphase chromosome movements.

Danlasky et al confirm the congression defects first described by Dumont et al, and they nicely quantify the congression defects in Figure 3. But Danlasky et al now argue that in fact kinetochore proteins are required for pulling forces on chromosomes that (i) promote kinetochore stretching early in meiosis I, with the presumably associated pulling forces possibly accounting for the congression defects; (ii) generate anaphase A chromosome movement toward the spindle poles during meiosis I, and (iii) are required to separate the homologs in bivalents during meiosis I anaphase. They also show that outer kinetochore proteins (KNL-1 and the MIS-12 complex component KNL-3) only partially dissociate from kinetochores during anaphase, with substantial levels remaining associated with kinetochores throughout anaphase during oocyte meiosis I, transitioning from kinetochore cup structures to ring structures that thus provide surfaces for possible attachment to microtubules both from the spindle poles and from the central spindle during anaphase chromosome movements. The authors provide the most impressive and highest resolution light microscopy imaging yet published of kinetochore protein localization and spindle structure during oocyte meiosis I in C. elegans (using in part a strain they previously generated with CRISPR to fuse GFP to both KNL-1 and KNL-3). Motivated by the Dumont et al observation that KNL-3 kinetochore localization does not depend on KNL-1, they also use CRISPR to degron tag both KNL-1 and -3 in the same strain, and knock down KNL-1 and -3 function simultaneously (both with auxin treatment for the degron tagged strain, and GFP RNAi for the strain in which both are tagged with GFP; these give nearly identical phenotypes). These improvements in methodology have led to substantial new insights that greatly advance our understanding of the requirements for outer kinetochore

proteins during C. elegans oocyte meiosis, and provide clear evidence that they have far more extensive requirements than has been previously appreciated. Perhaps the most compelling finding is that most homologs fail to separate after KNL-1/3 knockdown, with intact bivalents typically moving toward one pole (some bivalents moving to one pole and some to the other). Thus the anaphase movements that have been documented previously were in fact due to an extremely abnormal anaphase (without homolog separation), and/or perhaps also to less complete knockdown of kinetochore function (with RNAi targeting only KNL-1 or KNL-3 or other individual outer kinetochore components in previous studies). These findings provide a major correction to our understanding of the requirements for outer kinetochore proteins during oocyte meiosis I in C. elegans.

Danlasky et al also argue that highly redundant factors mediate microtubule/chromosome attachments to contribute to anaphase movements during oocyte meiosis I, based almost entirely on negative results involving the simultaneous knockdown of both KNL-1 and -3 and other oocyte chromosome or spindle associated proteins.

While Danlasky et al provide important new insights into the roles of kinetochore proteins (specifically KNL-1 and -3) during C. elegans oocyte meiotic cell division, the manuscript suffers from numerous problems that require extensive clarification before the manuscript can be considered suitable for publication in The Journal of Cell Biology. Indeed, the manuscript could possibly be greatly improved by eliminating much of the peripheral data and figures and focusing more narrowly on the positive results involving requirements for KLN-1 and -3. However, even these requirements require substantial clarification for readers to be able to critically evaluate the validity of the authors' conclusions concerning the requirements for KNL-1 and -3, and the manuscript fails to adequately describe the phenotype that results from the simultaneous knockdown of both KNL-1 and -3.

Major comments.

1. The authors do not adequately describe how their live imaging data were collected. In the Methods, they refer to 5, 10 or 15 second intervals but rarely specify what time intervals were used in the different figures. More importantly, they never state how many focal planes were collected in the z-axis for any of the figures, or how many of the focal planes were used for the images shown in the figures. They often do not state in the legends or elsewhere if maximum projection images are shown, or if the images were processed in other ways. The authors need to provide more detail on the imaging throughout the manuscript.
Information on the time interval between frames, the number of focal planes captured, the number of z-planes projected, as well as exposure times for each figure is now provided in the Materials and Methods. Only Fig S1A-B were deconvolved and this is now stated in the Materials and Methods.

2. There are numerous times in the manuscript when the authors do not provide

adequate information on the time points used for their quantifications. Without more such information, it is impossible to critically evaluate the validity of their conclusions, or the objectivity of their analyses. Examples of this include. The first examples apply to Figure 1 and in part their description on page 6, lines 3-7: the authors refer to metaphase and the "ring stage of anaphase" when describing the decrease in KNL-1/3 levels over time. What time points were used in the examples shown in Figures 1A? We have added this information to the legend. What time points were used for the calculation of the mean values? The "ring stage of anaphase" is especially vague, as the authors point out that one can detect rings over a substantial span of time during anaphase B. We have added the mean +/- SEM times relative to initiation of spindle elongation to the text. It also would be helpful if the authors could note the time points shown in Figures 1B and 1D. We have added “during spindle elongation” to the legend.

We have added a section on timing to the Materials and Methods that summarizes the sequential events of C. elegans meiosis to help the reader understand the timing in each data set.

3. In Figure 1, panels D-G seem largely peripheral to the roles of KNL-1 and -3 and the text describing them is very confusing. On page 7, lines 1-9, the authors repeatedly refer to various proteins or chromosomes occluding other proteins. What do the authors mean by occlusion? What do they mean by "spindle pole material?" What seems clear is that the rings form independently of the "linear elements" and independently of ROD-1 and ASPM-1. But what does occlusion mean or have to do with any of this? Proteins can diffuse and re-bind anywhere in a cell, and it is not at all clear how one protein (or chromosome) in one place might occlude another protein from being there (as far as I know, occlude just means block). And what kind of "optical artifact" are the authors referring to in line 9? This entire section and panels 1D to 1G seem tangential to the main points of the paper and the corresponding text is incomprehensible to this reviewer. These panels could easily be eliminated and the focus of the paper narrowed to more thoroughly and clearly explain the more important data and points.
We have changed the wording of this paragraph to hopefully make the description of the data more objective. Definition of occlusion: We observe “black holes” (a complete lack of fluorescence) that spatially correspond with DAPI staining or mCherry::histone H2B in C. elegans meiotic embryos with many different antibodies and many different GFP fusions. We interpret this to mean that the chromatin is so compacted that it excludes objects above some size threshold. Definition of spindle pole material: ASPM and NuMA/LIN-5 are two examples of proteins that are concentrated at the poles of spindles in many species independently of centrosomes. Many people like to think that these proteins are simply marking the location of microtubule minus ends. However, a BioRXiv manuscript from Stefanie Redemann’s lab shows that microtubule minus ends are distributed evenly throughout the spindle, so minus ends cannot explain the spindle pole localization of ASPM. One might think that dynein is carrying cargo along arrays of overlapping short microtubules to the “pole”. We have data that is inconsistent with this idea. We therefore have no idea what the atomic resolution structure of a spindle pole is. We still think this section is critical to show that rings of KNL-1,3 are not just due to

occlusion of spindle pole GFP::KNL fluorescence by highly compacted chromatin. We hope our revised paragraph makes this clear to the reviewer.

Also, in line 11, the authors refer to the time points 7:30 - 9:50 in Figure 2A, but the last time point in Figure 2A is 5:30.
The time points have been corrected in the text.

4. The conclusion that KNL-1/3 are required for kinetochore stretching (Figure 4) cannot be critically evaluated without more information as to how the authors made their measurements.
A section has been added to the Materials and Methods explaining the 2 methods we used.

First, how do the authors define metaphase for KNL-1/3 when their own data shown that chromosomes never align to form a metaphase plate? The Methods simply say that metaphase measurements were taken before spindle shortening began. How long before spindle shortening began (and also see major comment #5 below about spindle morphology)? The pre-anaphase measurements (presumably this should be called pre-anaphase A to be more clear) are said to be made between 90-120 seconds before homolog separation, with line scans used to assess separation. While this sounds reasonable, it would help to know how the authors chose 90 vs 100 vs 110 vs 120 for their time points.
The text now states “metaphase (5.7±0.47 min before initiation of spindle elongation) and pre-anaphase (2.2±0.15 min before initiation of spindle elongation)”.

Second, the authors measure two distances: homolog center to center, and outer kinetochore edge to outer kinetochore edge. The schematics show the homologs as nice perfect spheres, but in the images shown in Figure 4A the homologs are highly irregular in shape. How did the authors impose such perfect spheres onto these highly irregular shapes? How did the authors define the "edge" of the outer kinetochore cups? Additional details have been added to the methods section.

Third, the authors never refer to how the spindles might not be aligned along the plane that is parallel to the cover slips in their preparations. If the spindles are not parallel to the planes of the z-stacks, then the authors would need to use 3-D imaging and rotations to accurately measure the distances. The authors need to explain how they measured distance given the issue of spindle alignment relative to the plane of imaging. The following has been added to the Methods. “Only images in which both spindle poles and/or both bivalent halves were in focus were used for quantitative analysis. Both poles or half bivalents were considered to be in focus when both exhibited equal brightness and sharpness.”

The differences in these length measurements are very modest (though significant as shown), but without more information (and perhaps supplemental figures showing the examples used) it is impossible to judge the validity of the measurements and the differences. This is a key conclusion for the authors but the validity and objectivity of the

measurements cannot be assessed with the information given. We have given the requested additional information on both time points and the methods for measuring chromosome dimensions.

5. One last concern about the stretching data: the inference that the lack of stretching (if that is a valid conclusion) might explain the congression defects does not seem reasonable, given that the authors are measuring a process that occurs in control oocytes well after congression is complete
We now explicitly point to the fact that metaphase bivalent length is decreased in knl-1,3(kd) relative to controls and have softened the conclusion to: “reduced pulling forces could contribute to the congression defect”.

6. The authors do not adequately address the consequences of KNL-1/3 knockdown on spindle morphology, and in particular how normally or abnormally the mutant oocytes do or do not establish spindle bipolarity. The manuscript could possibly be much improved with more document of spindle bipolarity, using the GFP::ASPM-1 pole marker. The images shown in Figure 3A indicate some degree of spindle disorganization; in particular the middle example of the auxin treatment is highly irregular and not obviously bipolar. A single image of a partially bipolar spindle is shown using GFP:::LIN-5 as a pole marker. And in Figure 6 (panels B-E), it appears that nearly half of the spindles are abnormal in being "3-way" or "4-way", which seems to indicate tripolar or tetrapolar structures. Given that the authors rely on defining metaphase as being "before spindle shortening begins", the authors should more thoroughly document the impact of KNL-1/3 knockdown on spindle morphology over time, and document exactly how they can pinpoint when spindle shortening begins. Such information is critical to evaluating the validity of the measurements on kinetochore stretching and anaphase velocities. The manuscript would greatly benefit from more attention to this issue,
We have added an example z-stack through a GFP::ASPM-1 knl-1,3(kd) spindle as a Video and have added the text: “These congression defects were not due to a complete lack of spindle bipolarity as occurs in NDC80-depleted mouse oocytes (Gui and Homer, 2013; Yoshida et al., 2020). Z-stacks of 16/18 knl-1,3(kd) live metaphase spindles labelled with GFP::ASPM-1 exhibited two spindle poles (Video 2). 2/18 z-stacks exhibited a smaller third pole. “ Regarding the validity of spindle length measurements, the methods now state: “Only images in which both spindle poles and/or both bivalent halves were in focus were used for quantitative analysis. Both poles or half bivalents were considered to be in focus when both exhibited equal brightness and sharpness.”

rather than focusing on the curious business of occlusion and optical artifacts in Figure 1, the questionable/peripheral value of the him-8 analysis in Figure 7, and all of the largely negative (and due to unknown levels of functional depletion using RNAi/AID, entirely inconclusive) data on "highly redundant" factors contributing to chromosome movements/attachments in Figures 8-10 that is very much peripheral to the key points of the manuscript.
We now more thoroughly acknowledge the possibility of incomplete depletion. We have shortened this section and changed the sub-title.
7. The data on KNL-1/3 being required for anaphase A but not anaphase B (Figure 5) is

compelling but again the authors do not provide adequate information for critical evaluation of their conclusions, as to the time points being used for these velocity measurements. In panels 5G, 5G and 5I, the authors show data from representative oocytes. How did the authors define t = 0?
The legend now states that time zero is the initiation of spindle shortening which we have previously shown is the first APC-dependent event. This is nowexplained in the timing section of the Materials and Methods.
What is the relevance of the double arrows to the velocity measurements? The legend says they "indicate the initiation of anaphase chromosome movement relative to spindle shortening/elongation cycle." I have no idea what that sentence actually means. Does it refer to anaphase A or B? To when spindles begin to shorten, or begin to lengthen, or what? And what do those double arrows have to do with how they actually measured velocity for either A or B? It would be much more helpful if the authors could simply indicate with arrows and arrowheads what time interval was used for A and what interval for B in those examples. Maybe similar plots could be shown in supplemental data for the other oocytes. The legend says that anaphase A was measured over the 80 seconds before the beginning of spindle elongation, so that beginning could easily be shown with an arrowhead. Similarly, for anaphase B velocity (Figure 6A), the authors could indicate what interval constitutes "spindle elongation".
We have added the following to the legend for Fig. 5: “Time 0 is initiation of spindle shortening. Double arrows indicate the initiation of homolog separation in G, I and initiation of bivalent separation in H. Arrowheads indicate initiation of spindle elongation.” We have also added a section on timing to the materials and methods to justify our use of the initiation of spindle elongation as a reference point for comparing control with *knl-1,3(kd)*.

8. Another clarification on anaphase A and B movements concerns the authors documentation of "pop-out" chromosomes (Supplemental Figure 2C), also described as 3-way and 4-way spindles in Figure 6C and 6C. When the authors measured anaphase B velocities, did they only use bivalents that were not at the spindle edges, o pop-out, or 3-way, or 4-way? The bar graph in Supplemental Figure 2C indicates that about 50 of the bivalents were "pop-out". How did the authors judge if a spindle had a "pop-out" structure and which spindles were used to assess anaphase velocities?
We have changed “Pop-out” to “3 way or 4 way anaphase” in Fig. S2C to match the nomenclature in Fig. 6. Example images of 3 way and 4 way anaphase are shown in Fig. 6C and D and are called by these names in the Figure 6 legend. Fig. S2C has a cartoon drawing of a 3 way anaphase. We have added the following to the Materials and Methods: “Anaphase B velocities in Fig. 6A and Fig. S2C included only chromosomes separating parallel with the main spindle axis. Chromosomes segregating oblique to the main spindle axis in 3 or 4 way anaphases were not included in these figures, however, the velocities of these oblique anaphase B movements were not significantly different than the displayed values.”

9. One final clarification on anaphase chromosome movements. It is very intriguing that intact bivalents move together to a single pole after KNL-1/3 knockdown. But the authors do not devote any discussion to how this movement is being promoted. Indeed

in Figure 5C, there appears to be very little detectable tubulin inbetween the segregating pair of bivalents. The authors should provide some discussion as to how they think these anaphase B movements are being promoted.
Figure 5C is GFP::ASPM-1 and not GFP::tubulin. We feel that we already clearly stated that microtubule bundles appear to be pushing on the inner faces of chromosomes. As far as speculation as to the molecular mechanism of the elongation of microtubule bundles, this has been addressed in Dumont 2010 and Laband 2017. We chose to focus on pulling mechanisms in this manuscript.

10. The authors conclude that bivalents remain intact after KNL-1/3 knockdown due to a lack of pole-ward pulling forces early in anaphase. To support this interpretation, they show that both AIR-2/Aurora B and Separase show roughly normal dynamics in their localization after KNL-1/3 knockdown. An alternative interpretation is the KNL-1/3 are in fact required for cohesin cleavage, even though AIR-2 and Separase are present in roughly normal locations. Indeed, it is striking how normal the bivalents appear even after moving substantial distances toward a pole. One might just as easily argue that the homologs remain paired due to a failure to cleave cohesins and that KNL-1/3 are somehow required for this processing. The authors should at least acknowledge this alternative view and perhaps discuss how the bivalents could remain so normal looking in their morphology if cohesins have been cleaved. One additional piece of information that might help in evaluating this alternative explanation would be to know if the homologs and sister chromatids do come apart during anaphase of meiosis 2.
We have added text data and a Video showing that bivalents that end up at one pole come apart before metaphase II. We have added the possibility that KMN is a positive regulator of separase to the text and cite the opposite finding in Drosophila oocyte meiosis where NDC80 is a negative regulator of separase.

11. The data in Figures 8-10 and Supplemental Figures 4 and 5 seem very peripheral to the main points of the paper and while they involve some impressive genetic manipulations do not really contribute to understanding the roles of KNL-1 and -3. Moreover, the conclusions are very tentative, given that the depletions of the various proteins by RNAi or AID may well be incomplete with significant function remaining, a caveat the authors never even once acknowledge. Moreover, the authors devote an entire supplemental figure to KLP-7 localization and yet never knock down its function in the KNL-1/3 kd background. It seems the authors could drop these figures and publish them elsewhere, as they more distracting than helpful in this context, and the authors would be better served by address some of the clarifications that are needed and noted in the other major comments.
We now more thoroughly acknowledge the possibility of incomplete depletion. We have shortened this section and explain that knl-1,3,klp-7 triple depleted oocytes did not ovulate.
Minor Comments.

1. The authors note in the Introduction that knockdown of KNL-3 rescues anaphase in mutants lacking MEL-28 (Hattersley et al.., 2016). This is an intriguing observation that argue against a requirement for KNL-3 in anaphase forces, and the authors never return

to how they might explain this observation in light of their findings and conclusions about KNL-1 and -3 requirements. Indeed, the Discussion in general does not address very much of their own data and its relevance to the controversy over kinetochore function in C. elegans, but rather focuses extensively on spindle cutting studies and on the issue of redundancy of microtubule attachment to chromosome mechanisms, whereas the focus of the most important data in this paper is on KNL-1/3 function.
Hattersley 2016 was most likely looking at anaphase B, which is not blocked by knl-1,3(kd). Hattersley did not specify anaphase A vs B.

2. The Discussion also could be improved by including some reference to what is known about microtubule/chromosome attachments to chromosomes during oocyte meiosis in other organisms. The generality of the authors' findings with respect to oocyte meiotic cell division in other model systems is almost completely ignored.
There are a number of papers analyzing bivalent stretching or NDC80 localization in mouse oocytes that assume conventional pulling by kinetochores, however, we cannot find any published analysis of anaphase after KMN depletion in another animal oocyte system. We cite Gui and Homer, 2013 and Yoshida 2020 who showed that NDC80 is required for meiosis I spindle bipolarity in mouse oocytes. They found that meiosis II spindles were bipolar but did not analyze anaphase II, possibly because that would require fertilization or parthenogenic activation. We also cite Wang 2019 who knocked down the KNL-1 homolog, SPC105R, in Drosophila oocytes but did not analyze anaphase because it is technically challenging to bypass a natural metaphase I arrest in Drosophila oocytes (Kim McKim personal communication.) We do not specifically state that anaphase has not been analyzed after KMN depletion in the oocytes of another species because we cannot be absolutely sure this is true and because it would be impolite/obnoxious. The citations to Wang 2019 and Yoshida 2020, however, should help the reader in this regard.

3. Page 7, line 3: the authors refer to chromosomes being "embedded" in spindle pole material. What material are they referring to? Is it embedded or simply adjacent to it?
We have changed “embedded in spindle-pole material” to “surrounded by the spindle-pole protein, ASPM-1,…” to more objectively describe the results without over interpretation.

4. In Figure 2B, the authors might want to provide small arrowheads pointing to the fingers of KNL-1/3 projecting toward the poles.
Arrows have been added to the figure.

5. Page 10, line 4: the authors refer to Figure 4A-E; there is no E
We have changed the text to Fig. 4A-D.

6. Figure 5G-I: would it be possible to combine the data from all oocytes into one graph for each genotype, and show standard deviations?
No.
Or are the time courses highly variable from oocyte to oocyte?

Timing between individual events (like spindle rotation and initiation of homolog separation) have a reasonably small standard deviation. However, during all of meiosis, there is too much variability to overlay all plots. Again, more information on the spindle morphology over time, and the timing of events during meiosis I after KNL-1/3 knockdown might clarify some of the conclusions and arguments. Cell-cycle timing in knl-1,3(kd) was addressed in Vargas 2019. We have added a section on timing to the Materials and Methods.

7. The authors state that KNL-1/3 might be involved in the stretching of the X chromosomes in him-8 mutants. What happens if you knock down KNL-1/3 in the him-8 mutant background? We have conducted preliminary experiments. Unfortunately, in knl-1,3(kd), the congression defect makes it difficult to isolate the lagging univalent from the other chromosomes. Definitively addressing this will require chromosome paint technologies that are not yet working well enough for us in this system.

8. Page 14, lines 1-2: the authors should define "-1 oocyte" for a general audience
We have added a section to the Materials and Methods explaining what -1 oocytes are and why we used them in Fig. 8A-C.

9. Page 21, line 4: presumably the authors mean "non-NDC-80-dependent pulling forces persist..." and should make this more clear. However, again the authors fail to mention the caveat that their RNAi might not eliminate NDC-80 function and therefore they cannot conclude that other factors contribute to pulling forces.
The possibility of incomplete knockdown of NDC-80 is addressed in the results section. All references to possible redundancy have been rewritten.

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

The manuscript by Danlasky et at examines the forces driving chromosome poleward movement during anaphase in C. elegans meiosis. Experiments in many cell types reveal poleward segregation relies on kinetochore/microtubule dependent pulling forces. In contrast for C elegans meiosis, it has been proposed that anaphase A is driven by a kinetochore-independent microtubule-pushing mechanism involving microtubule associations with the inner chromosome face. However through a combination of cellular and functional genetic analysis, Danlasky and colleagues challenge this view. They find that the core kinetochore proteins KNL1 and KNL3 are required for anaphase stretching suggesting a role for these proteins in pulling rather than pushing. This conclusion is supported with fluorescent analysis of KNL localization. Overall the manuscript is well-written and the quality of the data is excellent. However there are a number of major and minor issues regarding the interpretation of the results and conclusions that must be addressed in order for the manuscript to be acceptable for publication:

Major:

1) The evidence for a conventional end-on "pulling" force driving chromosome separation is not compelling. The KNL-1,3 cups that are proposed to mediate this pulling force via potential end-on attachments also extend laterally around the chromosomes (there is also a high concentration of MTs that seem to be associating laterally here as well). Thus the defects observed upon KNL-1,3 knockdown could be due to diminished lateral interactions as opposed to end-on interactions. This isn't to say that there isn't end-on pulling, but the language used is fairly strong and doesn't seem to take into account these potential lateral interactions. I also think additional discussion is needed to reconcile the interpretation of a pulling force with the fact that severing MTs on the poleward side of chromosomes doesn't stop poleward movement in C. elegans. I know there is some discussion here, but the discussion really doesn't seem to address what is going on in anaphase C. elegans oocytes in particular, which appear to be a special case (as opposed to metaphase PtK2 cells).
"conventional" has been removed from "pulling" in the abstract. We have added more extensive discussion of lateral vs end-on attachments. We now describe several possible explanations that might resolve our results with EM and laser cutting results of others. We also specifically state that pulling is likely mediated by overlapping arrays of extremely short microtubules rather than a contiguous K-fiber. We don't actually know how much this makes C. elegans a "special case" because there are no EM tomograms from the majority (99.99999%) of species. 2) Stronger evidence is needed to demonstrate that the congression defect is truly unlinked from the separation defect. The mei-2 mutant is a good idea, but the congression defect does not seem to be as severe as the knl-1,3(kd) congression defect (i.e. no "stacked" bivalents). Along those lines, the mei-2 mutant does have some separation defects as well. So could the mei-2 be an intermediate between both? A more thorough characterization of the mei-2 congression defects (directly comparing to the knl-1,3(kd) congression defects would strengthen the authors' claims.
We have added quantification of the mei-2(ct98) congression defect next to the knl-1,2(kd) defect in Fig. 3C and now cite Fig. 3C in the text. The p value for the knl-1,3(kd) comparison with mei-2(ct98) was 0.13 by one way ANOVA. We now clearly state that stacking of bivalents could contribute to the segregation defects.

Other and related issues to be addressed:

Figure 1
-Could the GFP affect stability/protein turnover rate? (A-B)
-Stain endogenous KNL-1/3 (not GFP-tagged) still see same "ring structure" in anaphase B?
We now cite Monen 2005 which showed staining of untagged KNL-1 in rings during anaphase in a supplemental figure.
-Midzone microtubules would have - ends contacting segregating chromosomes? (C)
We do not want to specify whether plus or minus ends are contacting the inner face of chromosomes because a BioRXiv from Stefanie Redemann indicates that microtubules are extremely short and both ends are essentially everywhere at the light microscope

level. This is consistent with our EB1 imaging which looks identical to tubulin imaging. We do not know why ASPM apparently concentrates at only a subset of minus ends. We would like to avoid this quagmire in this manuscript.
-Why does Figure 1C look different than Figure 1F? - and seemingly discussed differently in the results as well (1C: ASPM-1 is surrounding KNL 1/3 rings (which in turn surround chromosomes); 1F: chromosomes are not surrounded by ASPM-1 due to spindle pole material)
During anaphase, ASPM-1 is brighter on the outer face of the chromosomes than on the inner face. If the contrast was jacked up on the anaphase spindle in 1F, the fraction on the inner face of the chromosomes would be visible. The anaphase in 1F is only shown as a positive control for the mei-2(RNAi) where there is no localized concentration of ASPM.

Figure 2
-Figure 2A does not have timepoints 7:30-9:50 as indicated in the text (pg 7, line 11)
The time has been corrected in the text.
Figure 4
-When is the timepoint for "metaphase" considered?
"5.7±0.47 min before initiation of spindle elongation" is now stated in the text.
-How many timepoints before initiation of chromosome movement is "pre-anaphase"?
"2.2±0.15 min before initiation of spindle elongation" is now stated in the text.
-A good control would be to show that the distance between the midzone-facing sides of homologs does not change between "metaphase" and "pre-anaphase"
The Materials and Methods state: "To ensure that homologs had not yet separated at the time of each pre-anaphase measurement, the ratio between peak mCherry pixel values and the pixel values of the trough between homologs was made for each bivalent. No significant difference in this ratio was found between metaphase and pre-anaphase or between control and *knl-1,3(kd)*."

Figure 5
-Congression defect in mei-2 mutant doesn't look as severe as that of the KNL 1/3 knockdown (i.e. chromosomes look closer to the metaphase plate and there doesn't appear to be the bivalent stacking as in the KNL-1/3 knockdown). Could a similar analysis be done as in Figure 3C for the mei-2 mutant? If the distribution is similar between the two mutants, it would strengthen the interpretation from this experiment.
Mei-2 data has been added to Fig. 3C and is not statistically different than knl-1,3(kd).
-as it is now, it looks like the mei-2 mutant has both a milder congression defect and a milder homolog separation defect
The difference between knl-1,3(kd) and mei-2(ct98) is not significant (revised Fig. 3C).
-Tying into the point above, does separation defect only occur when bivalents are stacked (as in Fig 5B, C)?
This is now addressed in the text. We also clarify that the homologs that do separate during anaphase A, do not undergo anaphase A separation.
-For 5H, instead of (or in addition to) measuring the distance between between intact bivalents, could the distance be measured between segregating homologs in the

population of homologs that do separate (~25?). Would they show anaphase A movement?
The text states: “These measurements included both distances between homologs that later segregated to opposite poles (Fig. S2B) and distances between stacked bivalents that later separated from each other intact (Fig. 5J).“ This is now re-stated in the discussion. We have also added this clarification to Fig. S2B.

Figure 8
-In text reference to 8D: "metaphase congression defects were observed in a significantly lower fraction of NDC-80-depleted embryos than in knl-1.3(kd) embryos" and gives some data, but the figure panel 8D doesn't contain any KNL-1,3(kd) data◊ was this from the same experiment in 3C?
We have changed the Fig. reference to 8D vs 3C.

Figure 9
-Parts of 9E-F are missing "n" values (blue control in E also has a discrepancy in number of points (9) and "N" value (10))
The Materials and Methods state:” For wild type-appearing anaphase B, only the number of embryos N is shown, since at this stage chromosomes group together and were treated as one moving mass.“ N has been changed to 9 to match the number of data points.

Figure S3
-Panel C, in texts states that the width difference between control metaphase and control pre-anaphase is significant, but this is not illustrated in the figure panel
We found that this width difference was actually not significant and have added the corresponding significance bar to Fig. S3C. To support the argument that bivalent length increases are due to stretching rather than ROD-1-dependent expansion, we now state that cross sectional area does not increase and the length still increases after rod-1(RNAi).
-Panel D, what does the blue dot mean?
The blue dot has been changed to green.
-Panel E, in text is highlighting no difference between metaphase v pre-anaphase, but in the panel is highlighting no difference between control and knl 1,3 kd area
We have changed the significance bars in Fig. S3E accordingly.
Figure S1
-Would be helpful to see the whole cell at metaphase in addition to the panels showing only one bivalent (Panels A-D)
-E-F, separase localization looks different between control and RNAi images (control: separase localization is perpendicular to division axis; RNAi: separase localization is parallel to division axis) why? Is this difference representative?
We have substituted examples which are more closely matched as far as the stage of meiosis and which look more similar. SEP-1 changes localization during anaphase and n is too small to conclude whether there might be a qualitative difference. We only state that the localization “was indistinguishable”.

Figure S4
-Panel B, in text suggest that AIR-2 dissociates from chromosomes in anaphase similarly between knl knockdowns and controls, but it looks like there is still AIR-2 localization on chromosomes in anaphase B (5:10 panel)
The text states: “AIR-2 also dissociated from chromosomes and associated with midzone microtubules during anaphase in knl-1,3(kd) spindles as it did in controls”. At 7:30 in the control and at 7:00 in knl-1,3(kd), AIR-2 looks identical which justifies our text description. At 4:50 in control, you can clearly see an intermediate in which AIR-2 rings elongate into rods or tubes (described in Dumont 2010). We can only see this intermediate in perfectly angled control spindles that are very close to the coverslip and therefore cannot conclude whether this is different in knl-1,3(kd).

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

This manuscript by Danlasky et.al. addresses the role of kinetochores during C. elegans female meiosis. This is an important area of investigation because previous work by others suggested that chromosome segregation in this system is kinetochore-independent. The authors demonstrate that following kinetochore depletion (via degron-mediated depletion of KNL-1 and KNL-3), chromosomes fail to properly align and orient on spindles, consistent with previous work. Moreover, they also go on to demonstrate that there are severe segregation defects in anaphase following kinetochore depletion. These authors previously showed that chromosomes stretch at the metaphase-to-anaphase transition, suggesting the existence of pulling forces at that stage, and in the current manuscript they demonstrate that this pulling is kinetochore-dependent. Interestingly, the authors also demonstrate that homologs fail to come apart in kinetochore-depleted anaphase, and instead move to spindle poles as intact bivalents. These findings demonstrate that, in contrast to previous studies, kinetochores do play important roles in chromosome segregation in C. elegans oocytes.

This manuscript reports important findings that have the potential to significantly advance thinking in this field. However, as detailed below, in some cases the data in the manuscript is not strong enough to support particular conclusions. I would like to emphasize that the extensive nature of the comments below is my attempt to improve a manuscript that I think could make an important contribution to the field; please do not interpret the length of my comments as an attempt to reject it outright.

Major points

1. The evidence that bivalents stretch at the metaphase to anaphase transition, and that this stretching is dependent on KNL-1/3, is strong. This data nicely supports the idea that there could be end-on pulling at this stage to mediate anaphase A, as the authors suggest. However, in other parts of the manuscript, the authors suggest that end-on attachments exist at other stages, but these claims are not well supported in light of previous work in the field; if the authors want to make these claims and refute previous studies, their data needs to be stronger and more convincing. Figure 2 in particular

seems to be used to suggest that there could be end-on kinetochore attachments in metaphase, but this is not convincing. Low resolution live imaging cannot provide information about whether there are end-on attachments to chromosomes. In Redemann 2018, they specifically look for microtubule ends associating with the ends of bivalents, within 250nm of the chromosome surface (this is the ribosome-free zone that has been defined as the kinetochore in EM images). In metaphase, they found very few microtubules in this zone (the ones they found looked to be predominantly laterally-associated, and they did not find evidence for end-on attachments (Figure 1C, S1 of that paper).

In the revision, we more clearly state that data in figure 2 might indicate that KMN might extend further poleward than the ribosome-free zone analyzed by Redemann 2018. We more thoroughly incorporate the lateral microtubule attachments observed in EM by Redemann 2018 and have softened conclusions about end-on attachments.

Redemann also looked at mid-late anaphase and also did not see a major population of microtubules on the outside surfaces of chromosomes (Fig 1D, 1E, S2). I don't think that the data presented in Figure 2 is strong enough evidence to refute this published data and to suggest that there may be end-on pulling interactions in metaphase. However, I do think that it is possible that upon spindle shrinkage at anaphase onset, transient end-on kinetochore attachments may form that could exert pulling force. This is better supported by the authors' data because they see chromosome stretching/narrowing at that stage (which nicely supports the idea of "pulling" at that specific stage) and also because that stage has not been described by EM - the spindles published are either in earlier anaphase, or at the end of anaphase A, when these transient end-on attachments could have disassembled. I think that the data in this manuscript is consistent with a lack of end-on attachments prior to anaphase, the establishment of end-on attachments at the metaphase-to-anaphase transition to mediate the stretching/pulling of chromosomes (and anaphase A), and then a switch to anaphase-B spindle elongation as the major form of segregation. This view better aligns with other published work in the field.

Since I don't think that this manuscript provides strong evidence for end-on attachments prior to the metaphase-to-anaphase transition, I think that the authors should reconsider the conclusions drawn from Figure 2, and revise strong statements proposing pulling forces prior to anaphase onset throughout the manuscript. As an example, the authors propose that the congression defects following KNL-1/3 depletion are due to lack of poleward pulling forces on chromosomes; this statement should be removed or softened since these proposed prometaphase pulling forces would presumably be from end-on attachments, which the data in the paper does not convincingly demonstrate are present.

We more thoroughly incorporate the lateral microtubule attachments observed in EM by Redemann 2018 and have softened conclusions about end-on attachments. In the discussion we point out that, in vitro, NDC80 only generates force at the end of a microtubule and suggest ideas that might resolve this with the EM of Redemann 2018.

I also suggest that the authors consider and discuss the ideas proposed in Davis-Roca 2017, which hypothesized that there could be a transient acquisition of end-on attachments to mediate chromosome stretching/anaphase A. That paper proposed that the pulling forces were transient, and under normal conditions (when the majority of

kinetochore signal dissociates from chromosomes), these end-on attachments would not persist. However, they also found conditions where kinetochore proteins remained brightly on chromosomes, and suggested that under those conditions the end-on attachments/pulling forces could remain. I encourage the authors of the current manuscript to consider whether the findings of Davis-Roca 2017 could fit into their thinking/model. Is it possible that any of the conditions examined (e.g. him-8 mutant with univalents) are conditions where kinetochore proteins are retained more strongly on chromosomes, to keep strong end-on attachments that mediate pulling forces (whereas usually these pulling forces would dissipate when the levels of kinetochore proteins are decreased)? We have added Fig. 7B showing no difference in KNL-1::GFP+GFP::KNL-3 fluorescence intensity between control and him-8. Our data shows that GFP::KNL intensity drops during anaphase to the same extent in him-8 as in controls. In Davis Roca 2017, the error detection system (as assayed by AIR-2 rings in mid anaphase) was activated by a 5-10 min incubation at 25°C as strongly as it was induced by him-8 (Fig. 1C, 2D and Methods in Davis-Roca). The kinetochore retention phenotype was not quantified in Davis-Roca 2017 and was not demonstrated for him-8. The temperature of our filming was 22-24°C, a temperature range with 100% hatch rate, so it is likely that the error detection system reported by Davis Roca was fully activated in all of our control and him-8 conditions. This interpretation would fit better with the Redemann EM data that did not find evidence for end-on attachments in mid-late anaphase under normal conditions. Unless Redemann et al. kept their worms at 15°C continuously during preparation or EM, the Davis-Roca error detection system was likely activated.

2. The evidence presented that KNL-1/3 depletion blocks separation of homologs in anaphase is very strong. However, the interpretation of this result by the authors (that homologous chromosomes need to be pulled apart by KNL-1/3 dependent forces, otherwise they behave as a single intact unit) is difficult to reconcile with previous studies in the field. If I understand the model presented in this paper correctly, bipolar pulling forces mediated by end-on attachments to kinetochores are required to pull chromosomes apart in anaphase A, and this pulling is required for bivalents to come apart in anaphase. However, there are many other mutant conditions where there are not bipolar pulling forces in anaphase A, and chromosomes are still able to separate from each other. For example, chromosomes still come apart during anaphase on monopolar spindles (they move to the same pole, but not as an intact unit), and chromosomes also segregate following depletion of KLP-15/16; in both of these mutant conditions there is no bipolar spindle at anaphase onset to exert Anaphase-A-like bipolar pulling forces.
In knl-1,3(kd) embryos, some homolog pairs separate properly during anaphase B. We cannot find any published quantitative data on homolog separation in klp-18(RNAi) or klp-15,16(RNAi). We cannot find any data in Mullen and Wignall, 2017 demonstrating that 100% of homolog pairs separate during anaphase I in klp-15,16 depletions. There is no analysis of bipolar pulling forces (stretching) in Mullen and Wignall, 2017. Muscat 2015 reported the following for klp-18(RNAi) anaphase: “in Meiosis I the six bivalents move away from the central pole, oscillate, and then coordinately move inwards to the pole”. It was then reported that 12 chromosomes were present in meiosis II but there

was no quantitative data presented showing when this separation occurred. We have added data to this revision showing that knl-1,3(kd) bivalents that segregate intact, eventually come apart before metaphase II. We have also added additional discussion of different reasons why homolog separation might be delayed in knl-1,3(kd).
In my mind, this makes it more likely that KNL-1/3 depletion is affecting the actual physical separation of chromosomes (e.g. by affecting separase cleavage of cohesin), instead of the interpretation presented in the paper (that bipolar pulling forces are required for individual chromosomes with cleaved cohesin to move to opposite poles). Although the authors show separase staining following KNL-1/3 depletion in Figure S1F, the anaphase picture is very messy, and does not show early anaphase (the chromosomes have already moved far apart), so this data does not clearly demonstrate that separase localizes normally to the midbivalent at the stage when it would be expected to cleave cohesin; providing more convincing evidence that separase localizes normally at anaphase onset is essential if the authors want to make this claim. We have replaced the anaphase example images of SEP-1 staining in Fig. S1. However, even if the authors could provide this evidence that separase localized normally, this would not serve as proof that cohesin was properly cleaved, so this should be taken into account in any re-writing of the narrative.
Therefore, I would encourage the authors to consider and discuss alternate hypotheses that could explain their results, and to discuss their result in the context of the other previous findings in the field. Also, for this manuscript, it is important for the authors to demonstrate whether chromosomes EVER separate from one another in KNL-1/3 depletion, by analyzing MII. This could shed light on whether homologs dissociate from one another (via cohesin cleavage) but move together to the same pole (i.e. they separate, but kinetochore pulling is required to get them moving in opposite directions), or whether homologs do not come apart (Movie S5 progresses to MII, but it was hard for me to determine if the bivalents ever dissociated into individual chromosomes). The latter result would be more consistent with a model in which KNL-1/3 depletion alters chromosome structure in a way that prevents homolog dissociation. The authors show in Figure 10 that KNL-1/3 depletion removes the midbivalent population of KLP-7; this suggests that there are some changes in the middle region of the bivalent following depletion of kinetochore proteins, which could also affect homolog dissociation. This possibility should therefore be discussed.
We have added text data and Video 6 showing that bivalents that end up at one pole come apart before metaphase II. We have added discussion of the possibility that KMN is a positive regulator of separase to the text and cite the opposite finding in Drosophila oocyte meiosis where NDC80 is a negative regulator of separase.

Other points:

- Page 6 lines 14-15: The statement that ASPM-1 surrounds the kinetochore rings is not clear from the data presented in Figure 1C. ASPM-1 appears enriched on the outside of the chromosomes, but if there is an enrichment on the inside surface of chromosomes it is faint and very hard to see. If the authors want to make this claim they should present more convincing data.

All of the authors of this manuscript can all see that GFP::ASPM-1 is surrounding the KNL rings in Fig. 1C. It is brighter on the outer face. This has also been previously described in McNally et al. 2016 Fig. 5B.

- The authors talk about the fact that kinetochore rings could provide a means of attachment to the inside surfaces of separating chromosomes (page 6 lines 12-14; page 7 lines 9-10; page 22 lines 8-11). However, it is my understanding that in the Laband/Dumont model, the ends of microtubules that are pushing on the inside surfaces of chromosomes would be predicted to be minus ends, not plus ends (plus end polymerization in the center of the spindle would provide force for the minus ends to push on the chromosomes). Therefore, the suggestion that the kinetochore rings could provide a means of end-on attachment to microtubules on the inside surfaces of chromosomes is confusing to me. Is there any evidence that kinetochores can make end-on attachments to microtubule minus ends?

Laband et al claimed to be able to distinguish plus from minus ends by flared morphology but did not provide data showing any specifity for plus or minus ends terminating at the inner surface of chromosomes. In vitro, NDC80 and KNL-1 both bind to the sides of microtubules (NDC80 generates force by binding to the side of the microtubule near the end).

- I did not understand the sentence on page 7 lines 10-11 that cites Fig. 2A ("as further suggested by...). The timestamps noted in the figure callout do not exist in the figure (which only goes until 5:30). The reference to the time stamps has been corrected. Additionally, even if the timestamps were just typos and this callout is referring to some of the frames shown in Fig. 2A, these images are not at high enough resolution to draw conclusions about whether there are microtubule attachment points on the inside surfaces of chromosomes.

We have added “and by EM results (Laband 2017; Redemann 2018)”.

- Figure 2: Details of the quantification are not in the materials and methods. Are the images in Figure 2A and 2B max projections or single slices? Was the quantification done on single slices? “Single focal plane” has been added to both the legend and the materials and methods for Fig. 2.

- Page 9 lines 3-5 states that the bivalents on the outside of the spindle have "no apparent microtubule contacts on one or more sides of the bivalent", but the spindles in Figure 3A and B that are referenced have bivalents on the outside of the spindle appear to have contacts on the sides. I might be reading too much into the wording of the sentence, but do you mean that they don't have contacts on the microtubule ends? It would also be helpful to put arrows or arrowheads pointing to the bivalents you are referring to, to help the reader.

Arrows have been added and the confusing text has been deleted.

- Page 9 line 4 has a figure callout to Fig. 4 A-E, but there is no Fig. 4E

This has been changed to Fig. 4A-D.

- Page 9: It would be helpful to define "inter-homolog" and "intra-homolog" stretch the first time you use them, as these terms are not intuitive to the non-expert reader

We now reference the appropriate Fig 4 sub-panel that illustrates each type of measurement at the first use of these terms.

- Page 9 lines 9-13: the authors should remove or soften the suggestions that the microtubule channels are caused by exclusion of microtubules from bivalents. The authors use the data in Figure 2 to make this point (which, as discussed in major point #1, I do not find convincing). Moreover, they also claim that the channels could be gone following KNL-1/3 depletion because the bivalents would be smaller (since they lack proteins cupping the ends). However, Figure 4A shows that there are still cups of some proteins on bivalents following KNL-1/3 depletion, so it is not clear how much smaller the bivalents would be.

We have deleted the suggestion that channels are caused by the exclusion of microtubules from bivalents.

- Page 12 line 5: The sentence talks about fibers pushing on the inner faces of chromosomes, but in the case of KNL-1/3 depletion, the inner face is not exposed (since the bivalent is intact), so it would be the outer surface. Rephrase sentence to avoid confusion.

Changed to …"pushing chromosomes apart".

- Page 12 line 16: The phrase "pushing between chromosomes" is confusing, because it suggests chromosomes have separated. I think rephrasing to "pushing between intact bivalents" might prevent confusion.

We have made the suggested change.

- Figure 7: Although it is clear that KNL-1/3 are on univalents as they stretch, the images in Figure 7 did not look like stretched KNL-1/3 rings - it simply looks like these proteins are coating the univalent. I therefore suggest changing the wording of the header on page 12 line 19 (and other references to stretched rings) so the reader is not confused. I don't think this affects the point you are trying to make (that kinetochore proteins are retained), but it will better reflect the data.

As suggested, the word "ring" has been deleted in 2 places referring to the stretched X univalent.

- Page 12 lines 8-11: The authors appear to imply that the KNL proteins on the univalent are physically coupled to the KNL rings on the other homologs, but the images are not at high enough resolution to state this. Just because there are kinetochore proteins surrounding chromosomes and the chromosomes are close together does not mean that they are physically coupled. Therefore, the statement that the rings are contiguous should be removed.

This section has been deleted.

- Page 14 line 19: change "separation" to "velocity" since homologs don't separate in kinetochore-depleted spindles.

This change has been made.

- Page 16 line 23: "...must require attachment of chromosomes to the ends of elongating microtubule bundles...". It is possible that chromosomes could also associate laterally with microtubules. Change to "...ends or sides..."

This change has been made.

- Page 17 line 10: This line references the "AIR-2 ring", but the cited papers show that AIR-2 leaves the ring early and relocalizes to microtubules, while other components remain in the ring structure longer, so the reference to the "AIR-2 ring" is not accurate. Rephrase statement.

Davis Roca 2017 Fig. 1C, 2D and Methods show that in methanol fixed control spindles, the “Percentage of mid anaphases with AIR-2 in rings” is 8% if the embryos are maintained at 15°C and 35% if the temperature is raised to 25°C for somewhere between 5 and 15 min. The data and cartoon at the end of the paper suggest that the ring left behind is a normal intermediate and that the transition from the ring to the microtubules is delayed in error conditions. We are not 100% sure of the reviewer’s issue here since they make the opposite argument below. This section has been shortened and revised.

- Page 17 lines 11-14: The authors state that their live imaging shows that rings "remain attached to chromosomes" but this is not shown by the data. Just because the rings are near chromosomes is not evidence that they are attached (the images are not high enough resolution to show this). Also, in Figure S4C, chromosomes are not shown. Higher resolution imaging in the Davis-Roca papers suggests the rings are removed in early anaphase and can remain as intact units (see Davis-Roca 2018, Figure 2 as an example). The data in the current manuscript are not convincing enough to argue against the view that the rings dissociate from chromosomes at early anaphase.

We have removed wording about attachment or detachment of rings from chromosomes. We now state that we observe rings elongating and reference papers that also documented this ring elongation.

- Page 18 lines 7-10: I don't understand this sentence connecting the NDC-80 depletion results to a conclusion about ring elongation. Please rephrase/explain better.

We have deleted this sentence.

- Page 21 line 1: Given my concern in major point #1 above (that there is not convincing evidence that there are end-on attachments prior to spindle shrinkage), I would suggest rephrasing "...may increase during spindle shortening to mediate this increased pulling" to "...may be established during spindle shortening to mediate this pulling".

This sentence was already deleted in response to other reviewer comments so we have made the “may be established during spindle shortening” change to the sentence above this.

- Page 21 lines 2-4: I don't understand this sentence, please rephrase. How does the fact that chromosomes move to poles following NDC-80 depletion support the persistence of pulling forces?

This paragraph has been completely rewritten in response to other reviewer comments.

- Figure 10: Put a label on Figure 10B so that it is clear what is being depleted in the plus auxin condition (same comment for Figure 5B). Since you use a couple of degron strains in the paper (KNL-1/3, dynein), it will help to label this in every figure for clarity.

These labels have been added.

- Figure S2: in the graphs in S2B and D, there are 2 "Ns" listed for each ("N" and "n"). State in the figure legend the difference between these numbers.

The definitions of N and n have been added to the legend.

- Figure S4E: How was depletion of GEI-17 confirmed? Since it is labeled with GFP in a strain where the microtubules are also GFP-tagged, this is impossible to see in the images (and I didn't see any other attempt to confirm depletion in other figures). We did not confirm depletion of GEI-17. This “allele” has been used in papers from 2 different labs and we now state that results could be due to partial depletion.

Typos:
- Figure 8 legend: intensty should be intensity
corrected
- Page 9 line 8: duplicate references to Vargas 2019
corrected
- Page 21 line 12: experments
fixed

August 25, 2020

RE: JCB Manuscript #202005179R

Prof. Francis J McNally
University of California, Davis
Dept. of Molecular and Cellular Biology
One Shields Ave.
Davis, CA 95616

Dear Frank,

Thank you for re-submitting your work entitled "KNL-1 and KNL-3 are required for pre-anaphase pulling forces, anaphase A and homolog separation during C. elegans female meiosis." The revised manuscript has been evaluated by the same three reviewers as the original submission. You will see that there is a consensus among these experts that the revision is markedly improved, and two reviewers recommend publication. Reviewer #2 maintains that without direct experimental support for the role of end-on vs. lateral interactions, the impact of this work will be limited. However, we are convinced by the other two Reviewers that even with softened conclusions, your work significantly advances our understanding of poleward forces that act on meiotic chromosomes. Therefore, we are glad to provisionally accept the manuscript for publication as an article.

As you will see, Reviewers #2 and #3 have made several suggestions on improving the clarity of the text. We hope you will find these comments useful, particularly the notes on using consistent terminology and descriptions of division stages. Please include a detailed response to the reviewers' comments along with the revised text. We would also suggest that you use the final revision to make sure that proper credits are given to previous publications. The role of kinetochore during meiosis in C. elegans has emerged as a controversial subject with numerous publications and often conflicting hypotheses. Because of this controversy, it would be proper to ensure that references are as comprehensive and unbiased as possible. Specifically, I am somewhat surprised by the lack of Wignall and Vileneuve (2009) in the list of papers that demonstrated existence of lateral interactions during metaphase (p.4, lines. 12-14) as well as when you mention that Aurora B forms a ring (p.5, lines 7-8).

We would be happy to publish your paper in JCB pending final revisions as delineated above and those necessary to meet our formatting guidelines (see details below).

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

1) Titles, eTOC: Please consider the following revision suggestions aimed at increasing the accessibility of the work for a broad audience and non-experts. We suggest more clearly delineating the advance, which is important for general cell biology journal with a diverse audience like JCB.

Title: Evidence for pulling forces mediated by end-on attachments of microtubules to the kinetochore in C. elegans meiosis

Running title: Kinetochore-dependent pulling for meiotic homolog separation
(more precision here than the current running title is needed)

eTOC summary: A 40-word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person.
- Please include a summary statement on the title page of the resubmission. It should start with "First author name(s) et al..." to match our preferred style.
revisions are needed to meet this style

2) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends.
Please indicate n/sample size/how many experiments the data are representative of: S5D

3) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions in the text for readers who may not have access to referenced manuscripts.
- Please include a brief description of the basic genetic features for all C. elegans strains, plasmids and cells or database IDs (e.g., Addgene, Wormbase, etc.) if available -- even if the materials were gifted by other investigators or described in other published work.
- Please be sure to include sequences for all primers and oligos (e.g., sgRNA, siRNA, etc.) including negative controls.
- Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. imaging medium
e. Fluorochromes
f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, http://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

B. FINAL FILES:

Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

-- An editable version of the final text (.DOC or .DOCX) is needed for copyediting (no PDFs).

-- High-resolution figure and video files: See our detailed guidelines for preparing your production-ready images, http://jcb.rupress.org/fig-vid-guidelines.

-- Cover images: If you have any striking images related to this story, we would be happy to consider them for inclusion on the journal cover. Submitted images may also be chosen for highlighting on the journal table of contents or JCB homepage carousel. Images should be uploaded as TIFF or EPS files and must be at least 300 dpi resolution.

It is JCB policy that if requested, original data images must be made available to the editors. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original data images prior to final submission.

The license to publish form must be signed before your manuscript can be sent to production. A link to the electronic license to publish form will be sent to the corresponding author only. Please take a moment to check your funder requirements before choosing the appropriate license.

Thank you for your attention to these final processing requirements. Please revise and format the manuscript and upload materials within 7 days. If complications arising from measures taken to prevent the spread of COVID-19 will prevent you from meeting this deadline (e.g. if you cannot retrieve necessary files from your laboratory, etc.), please let us know and we can work with you to determine a suitable revision period.

Please contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

Alexey Khodjakov, PhD
Monitoring Editor, Journal of Cell Biology

Melina Casadio, PhD
Senior Scientific Editor, Journal of Cell Biology

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

The authors have thoroughly addressed the comments of all reviewers and in my opinion the revisions to the manuscript make the manuscript suitable for publication withour further revision required. The authors have clarified the methods, and they provide a much more complete discussion of their interpretation of the results and why they favor a role for pulling forces mediated by end-on attachments of microtubules to the kinetochore, while acknowledging caveats and possible alternative mechanisms. The results they report greatly advance our understanding of the requirements for kinetochore function during C. elegans oocyte meiotic cell division, which has been a controversial and challenging issue. The manuscript includes a great deal of high quality data that greatly advance the field and point to future areas of investigation.

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

I've gone through the paper and rebuttal letter several times. Overall, I think I am slightly disappointed because I would have liked to have seen 1) more experiments performed to support the authors' interpretations and 2) more organizational changes to improve the manuscript clarity. The softening of the conclusions with regards to the contribution of end-on MT attachments to pulling forces and bivalent stretching is now more congruent with what the results show. However this also reduces the impact and main thrust of the original version. In addition, the results section still contains a great deal of speculation concerning the contributions of end-on vs. lateral attachments that occurs in the Results section. This speculation is ore appropropriate for the Discussion section (where there is further discussion of this) to make it clear that these interpretations are only speculation and have no additional experimental evidence to back them up.

-I appreciate the inclusion of quantifying the congression defect for the mei-2 mutant. This strengthens the authors' claim that the homolog separation defects in the knl-1,3(kd) experiments are not simply due entirely to a general congression defect. However, as many of the bivalents that don't separate are either stacked or aligned improperly, it seems the congression defect cannot be ruled out entirely. It is good that it is acknowledged when the result is first discussed and also considered when discussing the interpretation of this result later in the manuscript.

-In addition, the inclusion of the information that homologs in "intact" bivalents moving with one pole eventually separated by metaphase II is a welcome addition. This strengthens the authors' claim that any defect in cohesin removal is likely not responsible for the failed homolog separation in the knl-1,3(kd) background.

-There are a couple instances in the Discussion section where a result is interpreted in language that suggests something different than the actual result. For example:
• "The finding that depletion of KNL-1,3 or NDC-80 eliminates the bivalent stretching that occurs between metaphase and pre-anaphase" (pg 20, lines 11-12) suggests that there is no bivalent stretching in NDC-80 (eliminates = completely removes). However, the Results section contradicts this ("Bivalent stretching in NDC-80-depleted embryos was intermediate between that of control and knl-1,3(kd) embryos" pg 15, lines 3-5), suggesting that there is still some bivalent stretching in NDC-80 depleted backgrounds. Perhaps "diminishes" would be a more appropriate word choice?
• "The observation that neither homologs that later separate during anaphase B nor bivalents that remain intact during anaphase B, undergo anaphase A movement toward spindle poles in knl-1,3(kd)" (pg 21, 12-13), but Figure S2B and 5J has some non-0 values for anaphase A velocities in the knl-1,3(kd) backgrounds, indicating that in some cases, there is some movement toward the spindle pole. Perhaps "attenuated movement" would be a more accurate description. (This phenotype is also described as "No movement" in the Results section)
These inconsistencies, though minor and most likely unintentional, do alter the way the sections that follow are read

Additional issues:

It would greatly improve the clarity of the manuscript if "metaphase" and "pre-anaphase" were clearly defined in the Results section when they are first discussed. To me "metaphase" and "pre-anaphase" intuitively cover similar periods of time prior to homolog separation (obviously, as

mentioned several times, the "metaphase" stage is in actuality separated from the "pre-anaphase" stage by minutes). The Materials and Methods section adds further information, for example stating "we used time relative to the initiation of spindle elongation to compare parameters between control and knl-1,3(kd)." However, this is still slightly unclear to me. Both metaphase and pre-anaphase times were displayed as an average and an SD. What would be the rationale for choosing a time point at one end of the SD range (e.g. 6.17 min prior to spindle elongation) vs. the other end of the range (5.23 min prior to spindle elongation)? Is there a particular cellular event that would lead to the selection of the first time point over the second for making these quantitative measurements? Either moving some of this from the Materials and Methods to the Results or more explicitly grouping these sections together in the Materials and Methods (for example, some aspects of how time points for the stages were chosen are discussed in the "Timing" section and others are appear throughout the "Bivalent Stretching Measurements" section) might allow the reader to more easily assess the interpretations of these experiments.

-As one of the other reviewers states, the last several figures (especially Figures 9-10) don't seem to add all that much to the main conclusions of the paper, and their inclusion somewhat detracts from the more impactful figures that appear earlier in the paper.

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

The revised manuscript by Danlaskey et.al. is greatly improved. The authors have made a major effort to address the concerns raised by the reviewers, and I am largely satisfied with the way they addressed my specific concerns, and I appreciate their efforts. In addition, as before, this manuscript reports interesting findings that I think advance the field.

There are only a few remaining concerns, which I think can be addressed by text changes.

Remaining point from the previous review:
- Although the authors did show different images of separase localization in Figure S1 as I suggested, these images still do not show that "separase relocalization between chromosomes at anaphase onset was indistinguishable between control and KNL-1/3-depleted spindles", as the authors state on pages 11-12. The presented images do not show that separase localizes to the midbivalent region normally "at anaphase onset" (the images are from much later), and so I still think it is possible that separase relocalization could be delayed, contributing to the fact that homologs do not come apart until late anaphase. I appreciate the authors acknowledging a possible effect on separase on page 21 line 22-23, but I still think the sentence that begins on page 11 line 22 should be modified. It is ok to say that separase eventually relocalizes between separating chromosomes, given the data presented, but it should be clearly stated that it is possible that the timing of this relocalization could be delayed.

Other points:
- Pg. 2 line 9: "did not move towards a spindle pole" is confusing wording. Maybe change to "did not move apart towards opposite spindle poles".
- Page 10 line 3: The section header "KNL-1...required for stretching of metaphase bivalents" does not match the data. The authors convincingly show that bivalents stretch after the spindle begins shortening (i.e. after APC activation, which would suggest that anaphase has been initiated), but it is less clear how much pulling force there is on bivalents prior to this stage, at what most people would think of as metaphase. I think this confusion could be avoided by simply removing the word

"metaphase" from this section header.
- Pg. 15 line 20: "only found kinetochore dynein" should be amended to "found kinetochore dynein", since the "only" implies that it was shown that dynein was not on bipolar spindles, which is not the case.
- Pg. 17 lines 18-20. The sentence "midbivalent rings elongate in microtubule-free channels between separating homologs....but then transfer to microtubules in late anaphase" is not accurate. Some midbivalent components (AIR-2, SUMO) transfer to microtubules in late anaphase as stated, but others do not (they simply leave the ring) - therefore the wording of "the ring relocalizes" is confusing. This can be easily fixed by changing the sentence to ""midbivalent rings elongate in microtubule-free channels between separating homologs....and then some components leave the ring and transfer to microtubules in late anaphase".
- Figure S3A: The authors have done a good job in the revision discussing that pulling forces could be generated by either end-on or lateral attachments, to address reviewer concerns. Given this, I suggest changing the phrase "end-on pulling forces" in Figure 3A and the Figure 3A legend, to just "pulling forces" to make it consistent with the rest of the manuscript.
- I appreciate that you now describe KNL-1/3 as surrounding late lagging univalents (rather than being in rings), but in Figure 7A and B, they are still labeled as rings, which is confusing. Change the wording (removing "rings") in the figure.

Typo
- Pg. 9, line 15: "outside edge the spindle" should be "outside edge of the spindle"

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ONE SHIELDS AVENUE
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Sept. 13, 2020

To: Alexey Khodjakov and Melina Casadio
From: Frank McNally

Please find attached our final revision. Your suggested title was way over the JCB 100 character limit. We have changed the title to: “Evidence for anaphase pulling forces during C. elegans meiosis”. As detailed below, we have attempted to address all of the editor’s and reviewer’s suggestions. We then further edited the manuscript to bring the character count below 40,000. I apologize for the delay. I have been having health issues and Fig. S1 was made in Inkscape, resulting in significant delays getting it into a JCB-accepted file format. Final figures have been uploaded as Adobe Illustrator CS5 files. If there are problems with these, I can convert them to .eps.

Sincerely,

Frank McNally
Professor

Response to editor and reviewer comments:
We have added the Wignall and Villeneuve reference to the lateral MT and midbivalent ring citations in the introduction. We have also added Kaitna 2002, Rogers 2002 and Dumont 2010 to the midbivalent ring citations. We have also added a reference (Fabig 2020) to the stretched X univalent present during spermatocyte meiosis because end-on attachments were reported from electron tomograms.

The editor's suggested running title was over the JCB character limit. We have shortened it to: "Kinetochore pulling for meiotic homolog separation"

We have added an eTOC summary to the title page.

We have added the number of embryos to Figure S5D.

Error bars have been described as mean +/- SEM in the legends for Fig. 2D, Fig. 3, Fig. 4, Fig. 5, Fig. 6, Fig. 8, Fig. 9, Fig. 10, Fig. S2, Fig. S3, and Fig. S4.

We have corrected all um to μm.

We have added a statistics section to Materials and Methods. We have added detail to the methods for anesthetizing and mounting worms for live imaging.

Reviewer 2 suggestions:
Page 20: "eliminates" changed to "diminishes".
Page 12: "No movement" changed to "movement was greatly reduced".

Page 21: "The observation that neither homologs that later separate during anaphase B nor bivalents that remain intact during anaphase B, undergo anaphase A movement toward spindle poles in *knl-1,3(kd)* suggests a lack of poleward pulling forces that persist during normal anaphase A." changed to: "The observation that both homologs that later separate during anaphase B and bivalents that remain intact during anaphase B, undergo attenuated anaphase A movement toward spindle poles in *knl-1,3(kd)* suggests a lack of poleward pulling forces that persist during normal anaphase A."

Explanation of the choice of time points for bivalent stretching measurements added to the Results on page 10: "We previously found that ZWL-1-labeled kinetochore cups stretch dramatically just before homolog separation (McNally et al., 2016). Because homolog separation is defective in *knl-1,3(kd)* embryos (see below), we measured the change in bivalent length between 5.7±0.47 min and 2.2±0.15 min before initiation of spindle elongation. Both inter-homolog (Fig. 4B, C) and intra-homolog (Fig. 4D) distances increased significantly between these time points (referred to as metaphase and pre-anaphase) in control embryos. In *knl-1,3(kd)* embryos, inter-homolog and intra-homolog distances did not increase between metaphase and pre-anaphase and these distances were significantly smaller than in control embryos (Fig. 4 A-D, Fig. S3A-D)." Note that this explanation, required by reviewer 2 and the editor, forces us to disclose results out of order in the text.

We have shortened the triple degron results in the last section.

Reviewer 3 suggestions:
Page 11: We have added the following qualification to our separase results. “Separase localization on metaphase chromosomes and between anaphase chromosomes appeared normal in KNL-1,3 depleted spindles (Fig. S1) although a delay in re-localization might not be detected.”

Page 10: Header changed to “bivalent stretching”

Page 16: We have moved the word “only”: “Previous studies of *C. elegans* mitosis (Gassmann et al., 2008) or meiosis (Muscat et al., 2015) found kinetochore dynein only in experimentally induced monopolar spindles.”

Page 18: We have added the requested: “and then some components transfer to microtubules in late anaphase”.

“end-on” has been removed from fig. S3 and the legend to fig. S3.

“ring” has been removed from Fig. 7 regarding the lagging X chromosome.