The initial expansion of the *C. elegans* syncytial germ line is coupled to incomplete primordial germ cell cytokinesis

Jack Bauer, Vincent Poupart, Eugénie Goupil, Ken C. Q. Nguyen, David H. Hall and Jean-Claude Labbé
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Original submission

First decision letter

MS ID#: DEVELOP/2021/199633

MS TITLE: Cytokinesis incompletion drives the initial expansion of the C. elegans syncytial germ line

AUTHORS: Jack Bauer, Vincent Poupart, Ken C. Q. Nguyen, David H. Hall, and Jean-Claude Labbé

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but recommend suggestions that would greatly improve the study. Reviewer 1's suggestion of acutely inhibiting the actomyosin ring genetically or pharmacologically to test the role of the ring in supporting the syncytial architecture is an excellent one and, dovetails with reviewer 3's suggestion of assaying for ani-2's role in the the intracellular bridge stabilization during germline formation, though the actual experiments performed to test each point these may be distinct. All three reviewers provide excellent suggestions on improving the manuscript, for example, reviewer 2 and 3 suggest slowing the movies to make the manuscript reader friendly, and reviewer 2 provides many pointers for improving the clarity of the manuscript as well as citations which need to be provided. If you are able to revise the manuscript along the lines suggested, I will be happy receive a revised version of the manuscript.

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

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost

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

Reviewer 1

Advance summary and potential significance to field

Bauer et al. follow up on previous findings showing that C. elegans primordial germ cells undergo an incomplete cytokinesis during embryogenesis, resulting in the formation of a stable actomyosin ring connecting the PGCs. Here, they characterize the properties of the stable ring in the context of the first germ cell divisions upon larval feeding. Their findings lead to a model whereby sequential incomplete cytokinesis of PGCs result in the formation of a shared cytoplasmic compartment termed the 'protorachis', which templates the ultimate germline architecture of the adult.

Syncytial germlines are common to animals and it is unclear how they form, so the topic of the paper is an important one, and there is excellent imaging to support the authors' model. My suggestions for improving the manuscript are limited although the addition of functional experiments demonstrating the importance of the actomyosin rings to rachis stability would add significantly to the manuscript.

Comments for the author

Major comments
While it seems likely that the observed actomyosin rings support the architecture of the rachis in L1, this was not demonstrated and would be easy to test. Genetic and/or pharmacological experiments should be added to acutely inhibit actomyosin in L1 and determine whether the protorachis collapses.

Other suggestions
1. The problem studied is a three dimensional one, yet understandably data was shown in 2D. A 3D rendering of PGCs, protorachis, and cytoskeletal bridges based on real data, and added to the supplement as a movie, would help readers visualize the organization of the germline in L1.

2. A connection between PGCs in wild-type L1 was previously demonstrated by Abdu et al 2016 using photoactivatable Rhodamine-dextran (Supplementary Figure 3).
This reference should be cited and the text amended to state that the experiments in the manuscript are consistent with this prior observation.

3. The authors describe the localization of many actomyosin components in L1 PGCs. Given the role of CYK-4 and ZEN-4 in cytokinesis but not lobe formation in PGCs during embryogenesis (Maniscalco et al, 2020), it would not be surprising if CYK-4 and ZEN-4 show a different localization pattern in the L1 germline than cytoskeletal components. In the images shown in Fig S1 this might appear to be the case. The authors should more thoroughly compare the localization of CYK-4 and ZEN-4 to the other cytoskeletal components to determine whether they have similar or distinct localization patterns.

4. Butuci et al. 2015 should be cited for asynchrony of PGC cell divisions.

5. The photoconversion experiments in L1 are really interesting, and raise the question of why mitotic regulators do not diffuse between the PGCs to synchronize mitoses if they are interconnected. Is it possible that there is a very brief period when the two cells are physically isolated (by constriction of the bridge)? If so, the exact time of photoactivation in the experiments would be critical, and there might be a subset of embryos in which there is no diffusion at least not initially. For the reader to assess this, the data from each of the 7 embryos imaged should be shown individually, either on the graph already shown in 4C or as an additional supplemental figure.
6. The specific transgenes used to label various subcellular structures should be indicated in the Figure legend or Results if it is not included in the Figure.

Reviewer 2

Advance summary and potential significance to field

The germline is an immortal cell lineage required required for the sexual reproduction of all animals. Understanding the molecular mechanisms regulating the proliferation and differentiation of germ cells is of intensive interest to developmental and reproductive biologists and (of course) to readers of Development. It is becoming clear that in most or perhaps in all (?) cases, the cell division process of primordial germ cells and their descendants is modified in a mechanistically mysterious manner such that cytokinesis is incomplete resulting in the retention of connections between germ cells (variously referred to as "ring channels" or "ring canals"). Incomplete cytokinesis is undoubtedly of functional importance and it enables germ cells to share biomolecules with each other. Incomplete cytokinesis also raises many fascinating questions, a few of which are discussed in this manuscript. For example, how is it that interconnected cells might display disparate behaviors (e.g., cell division vs. quiescence or life vs. death)?

This manuscript provides an important advance in understanding through its high resolution analysis of the incomplete cytokinesis of the primordial germ cells in the L1 larval stage of the nematode Caenorhabditis elegans. The authors use electron microscopy, time-lapse imaging, and dynamic photobleaching experiments to probe the incomplete cytokinesis process. The data are of uniformly high quality. They present evidence supporting a model in which the "old" stable ring channel is retained and a new one is assembled de novo. I could see why a reviewer might criticize the potential impact of the study because it is largely "descriptive" and does not address the underlying molecular mechanisms. By contrast, our viewpoint is that high-quality descriptive studies such as these are foundational for ultimately getting at the molecular mechanism. The work is beautiful and could be published with only minor modifications. The authors should consider the points below.

Comments for the author

Major Points
1. The title of the manuscript should be changed to reflect the findings in a less judgmental manner. No data is presented that incomplete cytokinesis "drives" the expansion of the germline—it certainly accompanies it but causation is not established in this investigation.

2. Lines 269-270. The statement, "we photoconverted Dendra2 in a PGC undergoing cytokinesis and measured the dynamics..." confused us. This statement seems to imply that the key experiment shown in Figure 4 was done once. However, the legend to Figure 4 indicates that the experiment was done 7 times. Instead of burying this in the figure legend, the suggestion would be to emphasize the replicability of this experiment in the Results and Discussion text.

3. While the movies are nice, they could be more reader friendly by: (i) slowing them down; (ii) adding captions; and (iii) adding interpretive drawings into the frames.

4. Could the authors discuss (or compare and contrast) the origin of the ring channels resulting from incomplete cytokinesis and those that connect to the germ cell cytoplasmic lobes?

Minor Points
1. Line 53. Please cite the nice recent study from Flo Marlow's lab published in Development on the incomplete cytokinesis of germ cells in zebrafish.

2. Lines 57–61. Please consider a rewrite for clarity. The sentence is confusing because it leads off with specific mechanistic examples only to conclude that the mechanism is unknown.

3. Line 64. Please cite Hall et al. 1999.

4. Line 67. Please cite the work of Wolke et al. (2007), a foundational study in C. elegans which coined the term "ring channels" and was also published in Development.
David Greenstein and Todd Starich

Reviewer 3

Advance summary and potential significance to field

In this study the authors use high resolution microscopy methods to show that stable intercellular bridges are formed after division of the primordial germ cells (PGCs) in the nematode worm C. elegans. They demonstrate that PGC divisions are incomplete and the cytokinetic ring migrates to the nascent rachis (the name the ‘proto-rachis’) where it integrates and to establish intracellular bridges connecting each PGC to a common cytoplasm.

Comments for the author

In this study the authors use high resolution microscopy methods to show that stable intercellular bridges are formed after division of the primordial germ cells (PGCs) in the nematode worm C. elegans. They demonstrate that PGC divisions are incomplete and the cytokinetic ring migrates to the nascent rachis (the name the 'proto-rachis') where it integrates and to establish intracellular bridges connecting each PGC to a common cytoplasm. This manuscript is clearly presented and offers an important advance in our understanding of the early steps in C. elegans germline development. I have a few suggestions to improve the manuscript that are listed below.

Suggestions
1. Is ani-2 required for stable bridge formation between PGCs? Given the previously published work on this gene at later stages of development it would be helpful to know if incomplete cytokinesis and stabilization of bridges is regulated by ani-2 from the beginning of germline development. It would also be useful to know if the membrane lobes observed between the PGCs are also dependent on ani-2, as might be predicted based on its antagonizing activity during ring closure.

2. Resolution of TEM images in Fig. 1 should be increased as they are pixilated in the PDF sent out for review.

3. Should slow down speed and, if possible, add arrows to indicate where the intracellular bridge is located between the two PGCs in movie 1. This would be helpful to orient the less experienced readers who may not be familiar with the anatomical features highlighted in the TEMs.

4. Can movies be provided for the photoconversion experiment in Fig. 1E? Would be nice to see the movement of fluorescent signal from one PGC to another.

First revision

Author response to reviewers' comments

Response to reviews
We thank the reviewers for their insightful comments and constructive criticism. We have made significant efforts to address each of them and changes are highlighted in the revised version of the manuscript, as per editorial request. However, we would like to clarify that we had initially written (and submitted) our manuscript in Report format, yet we were advised soon after submission that the text was significantly too long for this format and that it should be converted to an Article, with the idea that the manuscript would stay as is for the first submission (i.e. with the Results and Discussion sections combined) but that it would be fully reformatted should we be invited to resubmit. We have therefore moved and expanded certain sections of the manuscript to a new Discussion section but we did not highlight any change in this section, as it would essentially put it all in yellow. We have also divided the Results section into sub-sections, following guidelines for Research Articles.

Reviewer 1
Advance Summary and Potential Significance to Field: Bauer et al. follow up on previous findings

showing that C. elegans primordial germ cells undergo an incomplete cytokinesis during embryogenesis, resulting in the formation of a stable actomyosin ring connecting the PGCs. Here, they characterize the properties of the stable ring in the context of the first germ cell divisions upon larval feeding. Their findings lead to a model whereby sequential incomplete cytokinesis of PGCs result in the formation of a shared cytoplasmic compartment termed the 'protorachis', which templates the ultimate germline architecture of the adult.

Syncytial germlines are common to animals and it is unclear how they form, so the topic of the paper is an important one, and there is excellent imaging to support the authors' model. My suggestions for improving the manuscript are limited, although the addition of functional experiments demonstrating the importance of the actomyosin rings to rachis stability would add significantly to the manuscript.

Major comments
1. While it seems likely that the observed actomyosin rings support the architecture of the rachis in L1, this was not demonstrated and would be easy to test. Genetic and/or pharmacological experiments should be added to acutely inhibit actomyosin in L1 and determine whether the protorachis collapses.
Response: We fully agree with the Reviewer that, in principle, it should be easy to test the prediction that perturbing the activity of actomyosin regulators results in a collapse of the membrane partitions defining the proto-rachis at the L1 stage, and we indeed extensively worked toward this. However, as detailed below, we find that this is not achievable.

We first employed strains bearing fast-acting ts alleles of actomyosin contractility regulators (*cyk-4*, *zen-4*, *nmy-2*, *cyk-1*). Upshifting mutant embryos at 26ºC for short periods resulted in penetrant cytokinesis defects, as reported previously (Davies et al., Dev Cell 2014). However upshifting newly-hatched L1 animals to 26ºC, even up to 12h, did not result in any phenotype, and feeding upshifted animals did not preclude germ cell division in early larval stages (L2, L3). In these conditions, we observed clear partition collapses only when animals reached the late L4 stage. This observation (no phenotype in early larval stages, strong phenotype in late L4 and adult stages) was independently reported for ts alleles of *cyk-4* and *zen-4* (Lee et al., eLife 2018).

We also used RNAi to deplete gene products at the L1 stage: soaking unfed L1 animals for 24h in dsRNA targeting *nmy-2* or *ani-1* had no impact on proto-rachis organization nor PGC division, and a collapse phenotype was only observed when treated animals subsequently fed on OP50 reached the late L4 stage. This demonstrates that the RISC complex had effectively been engaged by dsRNA treatment at the L1 stage but that the phenotype did not manifest itself until later in development. We repeated this experiment in mNG::ANI-1 expressing animals in which we also crossed *rrf-3(pk1426)* to make them hypersensitive to RNAi, yet obtained the same result: soaking these unfed, RNAi-hypersensitive L1 animals for 24h in dsRNA targeting *rho-1* (which in our hands gives the most potent effect in the early embryo), *ect-2* or *ani-1* had no impact on proto-rachis organization, and subsequent feeding of these soaked animals on plates seeded with bacteria expressing dsRNA for these same genes did not preclude PGC division and only resulted in a phenotype at the late L4 stage. Furthermore, the fluorescence levels of mNG::ANI-1 in *ani-1*(RNAi) animals was no different than in RNAi vector- treated controls, even after the first PGC division. We know that RNAi-mediated gene perturbation has previously been achieved in early larval germ cells after treatment of animals at the L1 stage (Lara-Gonzalez et al., Dev Cell 2019), yet our results show that it does not work for contractility regulators.
When we initiated this project, we treated L1 larvae with Latrunculin to score the organization of the proto-rachis and did not observe any striking disorganization. At the Reviewer's suggestion, and with now more experience with analysis of the primordial germ line, we repeated this experiment, treating L1 larvae expressing mNG::ANI-1, TagRFP::PH and mCh::H2B for 3-5 hours with two doses of latrunculin, 25 µM and 100 µM (we note that injection of adults with 5 µM Latrunculin was reported to result in a collapse of membrane partitions by Priti et al. [Nat Commun 2018]). Treatments with both doses worked in L4 larvae and resulted in a collapse of the intercellular bridges, demonstrating that the drug is effective. However, no phenotype was observed when we treated L1 larvae, even at the higher dose: the PGCs and membrane lobes remained distinct and the levels of ANI-1 accumulation at the proto- rachis remained unchanged. We observed the same result when we treated L1 and L4 animals expressing LifeAct::mKate2:

intercellular bridges were collapsed and mKate2 signal was greatly decreased in L4 animals, but not in L1 animals.

Overall, these results revealed that we cannot perturb actomyosin contractility function in PGCs at the L1 stage using ts alleles, RNAi or Latrunculin treatment. We have no idea why that is. One possibility is that actomyosin regulators within the proto-rachis are organized in a very compact and/or stable manner that makes perturbation difficult, a situation perhaps analogous to microtubule organization at the midbody prior to abscission. In support of this possibility, we measured the maximum fluorescence recovery after photobleaching of FP- tagged ANI-1, NMY-2 and CYK-7 in the proto-rachis of L1 larvae and found that it is less than 50%, which is comparable to the values measured at intercellular bridges of adult animals by Priti et al. (Nat Commun 2018). Another possibility is that all three approaches are ineffective for a different reason. Yet the end result is the same: we cannot easily perturb the activity of actomyosin regulators at the L1 stage and thus test whether they are functionally relevant to maintain the structure of the proto-rachis. We note that actomyosin contractility regulators are essential for embryogenesis, which precludes us from experimental designs in which we deplete them in parents and monitor phenotypes in their offspring. We hope that the Reviewer will agree that we worked extensively to try to answer this point and while we cannot reach a conclusion from these experiments, it does not weaken any of the conclusions that we put forth in our manuscript.

Other suggestions
2. The problem studied is a three dimensional one, yet understandably data was shown in 2D. A 3D rendering of PGCs, protorachis, and cytoskeletal bridges based on real data, and added to the supplement as a movie, would help readers visualize the organization of the germline in L1.
Response: We have used our EM data to produce a 3D rendering of the primordial germ line and added this as supplemental movie (Movie 3). This rendering indeed makes it easier to visualize the proto-rachis (white compartment), to which both PGCs (red, with nuclei in blue) and membrane lobes (magenta) are connected via intercellular bridges (yellow spheres). The approach that we employed to produce this rendering is described in the legend of the movie.

3. A connection between PGCs in wild-type L1 was previously demonstrated by Abdu et al 2016 using photoactivatable Rhodamine-dextran (Supplementary Figure 3). This reference should be cited and the text amended to state that the experiments in the manuscript are consistent with this prior observation.
Response: The reference has been added and the text was amended to reflect this prior observation. The paragraph now starts with mention of this result and our experiments are presented as an independent validation of this finding. The conclusion of this paragraph has also been changed to take into account these prior findings. Finally this work is also acknowledged in the first paragraph of the Results section, where we refer to previous observations of the complex membrane organization found between the two PGCs.

4. The authors describe the localization of many actomyosin components in L1 PGCs. Given the role of CYK-4 and ZEN-4 in cytokinesis but not lobe formation in PGCs during embryogenesis (Maniscalco et al, 2020), it would not be surprising if CYK-4 and ZEN-4 show a different localization pattern in the L1 germline than cytoskeletal components. In the images shown in Fig S1 this might appear to be the case. The authors should more thoroughly compare the localization of CYK-4 and ZEN-4 to the other cytoskeletal components to determine whether they have similar or distinct localization patterns.
Response: We had not considered this possibility and, accordingly, we had not measured centralspindlin components in the proto-rachis. We have now done this for both mNG::CYK-4 and GFP::ZEN-4 and we find that their distribution at PGC intercellular bridges and at the base of lobes is similar to that of other contractility regulators: we find two peaks of fluorescence intensity bordering a minimum. Based on the work of Maniscalco et al. (2020), this finding would apparently argue against the possibility that the actomyosin rings at the base of lobes in the proto-rachis originate from those described during embryogenesis. However, it does not preclude that CYK-4 and ZEN-4 would not be required for lobe formation but would subsequently be recruited to some of the lobes' actomyosin rings after they have formed. We favour the latter possibility and discuss it in what is now the second paragraph of the manuscript's discussion. The results obtained from measurements of CYK-4 and ZEN-4 at intercellular bridges have been included in the manuscript (Figure 2D) and examples are shown in Figure S2D-E.

5. Butuci et al. 2015 should be cited for asynchrony of PGC cell divisions.
Response: The reference has been added and the text amended to reflect this prior finding.

6. The photoconversion experiments in L1 are really interesting, and raise the question of why mitotic regulators do not diffuse between the PGCs to synchronize mitoses if they are interconnected. Is it possible that there is a very brief period when the two cells are physically isolated (by constriction of the bridge)? If so, the exact time of photoactivation in the experiments would be critical, and there might be a subset of embryos in which there is no diffusion, at least not initially. For the reader to assess this, the data from each of the 7 embryos imaged should be shown individually, either on the graph already shown in 4C or as an additional supplemental figure.
Response: We had not considered the possibility that there may be a difference in the timing of diffusion initiation within our population, and we felt that having only 7 cases was perhaps too small to see whether this could be the case. We have therefore measured photoconverted Dendra2 diffusion in dividing PGCs of 5 additional L1 animals, increasing our sample size to n=12. To assess the possibility brought by the reviewer in a quantitative manner, we asked whether there were cases in which there was a delay between the first timepoint after photoconversion and the start of fluorescence decrease (in essence, the average fluorescence loss between the first and second acquired timepoints following photoconversion, which are separated by 15 sec). We found that fluorescence decreased in all cases, with an average loss of 6.6 ± 4.5% of fluorescence within the first 15 seconds after photoconversion. This is not different from what we measured in animals in which both PGCs are quiescent, where fluorescence loss was 5.9 ± 4.3% within the first 15 sec after photoconversion. These results demonstrate that diffusion within the primordial germ line occurs in a similar manner whether a PGC is undergoing division or not, and further supports the notion that the intercellular bridge remains open during PGC cytokinesis. These values are now reported in the last sub-section of the Results section.
Regarding the notion that cytoplasmic diffusion occurs between two PGCs, with one in mitosis, we agree with the reviewer that this is puzzling. Our hypothesis is that there is limited diffusion for some cell cycle or mitotic regulators, perhaps similar to the diffusion differences that have been reported for cell fate regulators at the 1-cell stage. This possibility, which is currently being investigated in the Labbé lab, is raised it in the last paragraph of the discussion.

7. The specific transgenes used to label various subcellular structures should be indicated in the Figure legend or Results if it is not included in the Figure.
Response: Each figure or figure legend now clearly indicates the fusion proteins that is being monitored.

Reviewer 2

Advance Summary and Potential Significance to Field: The germline is an immortal cell lineage required for the sexual reproduction of all animals. Understanding the molecular mechanisms regulating the proliferation and differentiation of germ cells is of intensive interest to developmental and reproductive biologists and (of course) to readers of Development. It is becoming clear that in most or perhaps in all (?) cases, the cell division process of primordial germ cells and their descendants is modified in a mechanistically mysterious manner such that cytokinesis is incomplete, resulting in the retention of connections between germ cells (variously referred to as "ring channels" or "ring canals"). Incomplete cytokinesis is undoubtedly of functional importance and it enables germ cells to share biomolecules with each other. Incomplete cytokinesis also raises many fascinating questions, a few of which are discussed in this manuscript. For example, how is it that interconnected cells might display disparate behaviors (e.g., cell division vs. quiescence or life vs. death)?

This manuscript provides an important advance in understanding through its high resolution analysis of the incomplete cytokinesis of the primordial germ cells in the L1 larval stage of the nematode Caenorhabditis elegans. The authors use electron microscopy, time-lapse imaging, and dynamic photobleaching experiments to probe the incomplete cytokinesis process. The data are of uniformly high quality. They present evidence supporting a model in which the "old" stable ring channel is retained and a new one is assembled de novo. I could see why a reviewer might criticize the potential impact of the study because it is largely "descriptive" and does not address

the underlying molecular mechanisms. By contrast, our viewpoint is that high-quality descriptive studies such as these are foundational for ultimately getting at the molecular mechanism. The work is beautiful and could be published with only minor modifications. The authors should consider the points below.

Major Points
1. The title of the manuscript should be changed to reflect the findings in a less judgmental manner. No data is presented that incomplete cytokinesis "drives" the expansion of the germline—it certainly accompanies it but causation is not established in this investigation.
Response: We have now changed the title to "The initial expansion of the *C. elegans* syncytial germ line is coupled to primordial germ cell cytokinesis incompletion", which conveys a notion that is comparable to the previous title but is less definitive regarding causation.

2. Lines 269-270. The statement, "we photoconverted Dendra2 in a PGC undergoing cytokinesis and measured the dynamics..." confused us. This statement seems to imply that the key experiment shown in Figure 4 was done once. However, the legend to Figure 4 indicates that the experiment was done 7 times. Instead of burying this in the figure legend, the suggestion would be to emphasize the replicability of this experiment in the Results and Discussion text.
Response: We corrected this confusion by making PGC plural, which conveys that the experiment was conducted in more than one cell, and adding n=12 in the text to clarify the number of times in which the experiment was conducted. Please note that we increased the number of cases (from 7 to 12) to better address point 6 raised by Reviewer #1.

3. While the movies are nice, they could be more reader friendly by: (i) slowing them down; (ii) adding captions; and (iii) adding interpretive drawings into the frames.
Response: The rate of all movies has been slowed down and additional information was inserted to clarify what is displayed. At the request of other Reviewers, we have also included additional movies that better document the 3D organization of the primordial germ line and the diffusion of Dendra2 in photo-conversion experiments.

4. Could the authors discuss (or compare and contrast) the origin of the ring channels resulting from incomplete cytokinesis and those that connect to the germ cell cytoplasmic lobes?
Response: We currently do not know what is the actual origin of lobes and speculate in the manuscript that they arise during interphase, in the process described by Maniscalco et al. (2020). Reviewer #1 suggested an experiment that enabled us to document one additional aspect of their composition (see point #4, above), and we now address their possible origin more thoroughly in the second paragraph of the manuscript's discussion.

Minor Points
5. Line 53. Please cite the nice recent study from Flo Marlow's lab published in Development on the incomplete cytokinesis of germ cells in zebrafish.
Response: The reference has been added.

6. Lines 57-61. Please consider a rewrite for clarity. The sentence is confusing because it leads off with specific mechanistic examples only to conclude that the mechanism is unknown.
Response: We have now changed the end of this sentence to convey that "our understanding of the molecular mechanism enabling cytokinesis incompletion in animal germ cells remains incomplete".

7. Line 64. Please cite Hall et al. 1999.
Response: The reference has been added.

8. Line 67. Please cite the work of Wolke et al. (2007), a foundational study in C. elegans which coined the term "ring channels" and was also published in Development.
Response: The reference has been added. We also included that stable germ cell intercellular bridges are also called ring channels to better link with this study.
Reviewer 3
In this study the authors use high resolution microscopy methods to show that stable intercellular bridges are formed after division of the primordial germ cells (PGCs) in the nematode worm C. elegans. They demonstrate that PGC divisions are incomplete and the

cytokinetic ring migrates to the nascent rachis (the name the 'proto-rachis') where it integrates and to establish intracellular bridges connecting each PGC to a common cytoplasm. This manuscript is clearly presented and offers an important advance in our understanding of the early steps in C. elegans germline development. I have a few suggestions to improve the manuscript that are listed below.

Suggestions
1. Is ani-2 required for stable bridge formation between PGCs? Given the previously published work on this gene at later stages of development it would be helpful to know if incomplete cytokinesis and stabilization of bridges is regulated by ani-2 from the beginning of germline development. It would also be useful to know if the membrane lobes observed between the PGCs are also dependent on ani-2, as might be predicted based on its antagonizing activity during ring closure.
Response: We completely agree with the Reviewer and we tried very hard to interfere with the activity of actomyosin regulators in PGCs but, for unknown reasons, this could unfortunately not be achieved. Please see Reviewer #1's comment #1 for more details on this matter and for new results following the use of latrunculin, which relates to this point.

2. Resolution of TEM images in Fig. 1 should be increased as they are pixilated in the PDF sent out for review.
Response: The resolution of these images has now been optimized.

3. Should slow down speed and, if possible, add arrows to indicate where the intracellular bridge is located between the two PGCs in movie 1. This would be helpful to orient the less experienced readers who may not be familiar with the anatomical features highlighted in the TEMs.
Response: We slowed down Movie 1 so that sections are easier to visualize. Arrows pointing to intercellular bridges can be found in the figures and therefore we have not included them in this movie. At the request of Reviewer 1, we have however used the EM data to make a 3D rendering of the primordial germ line (now Movie 3), which we hope will facilitate representation of the structure in 3D. Please see also Reviewer #2's comment #3.

4. Can movies be provided for the photoconversion experiment in Fig. 1E? Would be nice to see the movement of fluorescent signal from one PGC to another.
Response: Movies illustrating Dendra2 diffusion after photoconversion in quiescent (Movie 2) and dividing (Movie 6) PGCs have now been included.

Second decision letter

MS ID#: DEVELOP/2021/199633

MS TITLE: The initial expansion of the *C. elegans* syncytial germ line is coupled to primordial germ cell cytokinesis incompletion

AUTHORS: Jack Bauer, Vincent Poupart, Eugénie Goupil, Ken C. Q. Nguyen, David H. Hall, and Jean-Claude Labbé

I have received all the reviewer comments, and as you will see the overall evaluation is positive and we would like to publish a revised manuscript in Development. However, reviewer 1 highlights some textual edits which will greatly improve the clarity of the manuscript. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. I do not expect to send the study back to the reviewers, it is important that you make the suggested edits. Please highlight all the textual changes in the revised manuscript. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

Reviewer 2

Advance summary and potential significance to field

The authors have nicely addressed all the points raised in the prior reviews.
Movie 3 will be a big help to readers. The manuscript advances our understanding of how the C. elegans syncytial germline forms through incomplete cytokinesis. The authors should be congratulated on their fine study.

David Greenstein

Comments for the author

Minor points
1. Use first-stage larvae (lines 37, 72, etc.)
2. Line 53. Perhaps add zebrafish as per the new citation.
3. Line 59. Probably, "arise from" is preferable.

Reviewer 3

Advance summary and potential significance to field

See previous review.

Comments for the author

I am satisfied with the authors' efforts to address my concerns and believe they adequately addressed the concerns raised by the other reviewers. I have no further suggestions and recommend this article for publication.

Second revision

Author response to reviewers' comments

Reviewer 2 proposed three minor suggestions to improve the manuscript:

1. Use first-stage larvae (lines 37, 72, etc.)
Response: We have now changed all such instances (3) in the manuscript.

2. Line 53. Perhaps add zebrafish as per the new citation.
Response: The reference to zebrafish germ cells was added to the sentence.

3. Line 59. Probably, "arise from" is preferable.
Response: While we are not completely sure that we see where the reviewer suggests making this change, we think that it concerns the sentence in which we state that "... intercellular bridge stabilization was shown to rely on impaired recruitment of the abscission machinery..." and we consider that this sentence is correct. Furthermore the sentence before this one already uses the term "arise" and we prefer not to use the same term in consecutive sentences. Therefore no change was made.

Third decision letter

MS ID#: DEVELOP/2021/199633

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AUTHORS: Jack Bauer, Vincent Poupart, Eugénie Goupil, Ken C. Q. Nguyen, David H. Hall, and Jean-Claude Labbé

ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.