Drosophila sperm development and intercellular cytoplasm sharing through ring canals do not require an intact fusome

Ronit S. Kaufman, Kari L. Price, Katelynn M. Mannix, Kathleen M. Ayers, Andrew M. Hudson and Lynn Cooley
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First decision letter

MS ID#: DEVELOP/2020/190140

MS TITLE: Drosophila sperm development and intercellular cytoplasm sharing through ring canals do not require an intact fusome

AUTHORS: Ronit S. Kaufman, Kari L. Price, Katelynn M. Mannix, Kathleen M. Ayers, Andrew M. Hudson and Lynn Cooley

I apologise for coming back to you after a long delay, due to the difficult circumstances for all. 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.

The overall evaluation is clearly positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. 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.

Reviewer 1

Advance summary and potential significance to field

In most organisms, germ cells go through several rounds of incomplete mitosis forming cysts of cells, which remain interconnected by cytoplasmic bridges. It is often transient in females, whereas it is a well-known feature of spermatogenesis.
One hypothesis for its widespread occurrence in males is that sperm needs to share X-linked and Y-linked gene products for their differentiation, while X and Y chromosomes are segregated to different cells during meiosis. In this manuscript Kaufman and colleagues demonstrate that proteins can move between cells through these cytoplasmic bridges throughout male germ cells development in Drosophila. In addition, they analyzed putative functions of the fusome, which is germline-specific organelle, made of vesicles, and which links cells within each cyst.

Comments for the author

1) Quality:
1a) Experiments: experiments are well performed and convincing. The results are analyzed rigorously and conclusions are well supported by the results. The methods used here to demonstrate proteins movements (photoactivation and photobleaching FLIP) are proven and published before by the same lab in other cellular contexts (female germline development and somatic cells) (Airoldy et al, 2011; McLean, et al. 2013).
Knock down of the fusome using shRNA against a-Spectrin and hts are convincing.
It is nicely demonstrated by EM sections of mutant testis.

1b) Completeness: my major comment regards the completeness of the analysis of hts mutant phenotypes. The authors convincingly showed that the fusome is absent in hts mutant males, and do not detect any reduction in male fertility. They conclude that disruption of the fusome has no effect on spermatogenesis.
However, one major phenotype in hts mutant females is complete cytokinesis during cysts mitosis giving rise to cysts with low and abnormal number of cells between 2 and 8 cells instead of 16 cells (Yue and Spradlin, 1992). A similar phenotype is found in the absence of a-Spectrin (de Cuevas, et al. 1996). In hts females, most of these abnormal egg chambers lack an oocyte and are not fertile. However, in males, complete cytokinesis in pre-meiotic cells would not necessarily lead to sterility as partial cysts could enter meiosis independently and be fertile. My question is whether the authors have checked cysts integrity in hts mutant males. Are they all made of 16 cells? Have ratios of 2, 4, 8, 16 cells cysts changed? Are cysts divisions still synchronous?
I think it is very important to search for these defects, which may not lead to sterility or a reduction in fertility.

2) Impact:
2a) Novelty/fundamental/broad interest Movements of proteins between sister-cells in Drosophila males cysts is quite an expected result. It has been shown by the same lab in somatic cells and in female cysts. However, the demonstration is novel in males. These results and conclusions are likely to be conserved in other species, and are thus of broad interest.
The absence of defects following the disruption of the fusome is more surprising. However, it needs to be supported by additional experiments (see 1b).
2b) Extensibility The authors noticed that movements of proteins does not seem to depend on protein size. This point opens up future lines of research to understand the selectivity and mechanisms of protein sharing between cells.
Minor points: it is not always clear if the authors take into account the GFP tag when they analyzed protein size, as they only analyzed tagged versions of proteins.

Reviewer 2

Advance summary and potential significance to field

Germ cells have long been known to be interconnected by intercellular bridges, or ring canals, that enable clonally related to cells to develop as syncytial cysts. Ring canals have been proposed to

permit sharing of cytoplasmic components and signaling molecules needed to coordinate cell growth and differentiation. For example, ovarian ring canals in Drosophila are needed to allow directional transfer of proteins, mRNAs and organelles from nurse cells into the oocyte. In the male germline, ring canals are thought to be important for sharing of post-meiotically transcribed X-linked mRNAs that are needed at later stages of haploid sperm development. However, this has never formally been tested. In Drosophila, a specialized organelle, the fusome, passes through ring canals and is important during oogenesis for coordinating the cell cycle and establishing the identity of the oocyte during germline cyst formation. In Drosophila males, the fusome was recently shown to be important for signaling DNA damage in spermatogonia, leading to cyst elimination. However, the importance of the fusome in developing Drosophila male germ cells has not been rigorously tested.

Here, using a photoactivatable (PA) GFP, as well as various proteins traps and photobleaching approaches the authors examine the requirement for ring canals and fusome during male germ cell development. They show that ring canals permit sharing of soluble cytoplasmic factors throughout all stages of spermatogenesis, but that not all proteins pass equally to other cells within a cyst. In elongating spermatids PA-GFP passes first through the ring canals before spreading to other spermatids within the cyst, suggesting that ring canals are required for sharing of cytoplasmic factors. Interestingly, knockdown of fusome components has little effect on cytoplasmic sharing or on male fertility, although loss-of-function mutations in the fusome component hts result in a gradual reduction or loss of fertility, depending on the allele.

Overall, this study is beautifully done, the conclusions are well justified, and the data provide a significant advance in our understanding of the roles of ring canals and the fusome during male germ cell development.

Comments for the author

MAJOR COMMENTS
1. I am not sure I agree with the interpretation that the hts mutant phenotype indicates a role for hts outside the male germline, i.e., in the soma. Germline-specific knockdown of α-spectrin results in a significant reduction of the fusome, as seem by immunostaining and by TEM, yet the males are fertile. However, a small amount of Hts/Adducin protein is still present, and it seems possible that this could be sufficient to carry out a germline-specific function of hts that is potentially independent of the fusome per se. For example, the authors do not comment on whether Hts/Adducin is still present at the growing ends of the spermatid cysts following α-spectrin knockdown. The stronger hts mutants would presumably have less Hts/Adducin protein available to carry out this or any other germline-specific function of hts.

2. Along these lines, it would be helpful to provide an image showing the morphology of the clustered Hts/Adducin signals at the growing ends of late-stage elongating spermatid cysts (typically located between Zones 1 and 2) from the testes shown in Fig. 5A-A' and E-E", as it is not clear from the images provided whether the Hts/Adducin signal in α-spectrin knockdowns resembles controls at this stage.

3. The diagram in Fig. 4T is a bit misleading, as is does not indicate the obvious movement of the PA-GFP away from the ring canals within the spermatids in which PA-GFP has been activated (i.e., in the direction of the arrow pointing towards the "3" in Fig. 4M. Perhaps a skinnier arrow (or the same short arrow as in 4M) could be used to indicate that this also happens, as this movement is quite evident from the corresponding movie.

4. It is unclear why the GFP signal in Fig. S2 (1st panel, 2nd row) localizes to ring canals in this image. Is this because Pav-GFP is also being expressed in these cells?

MINOR CORRECTIONS
p. 3, 10th line from the bottom: delete "and" before "has been implicated"
p. 6, Table 1: change "eIF4AE1" to "eIF4E1"
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p. 14, 1st line: I believe this should say “unchanged” rather than “changed”
p. 15, Drosophila strains, 4th line: change “eIFα” to “eIF4A”; also, alleles should be italicized Fig. S1: change label “eIF4a” to “eIF4A”
Fig. S1 legend: should indicate that GFP and mito-GFP are not FlyTrap lines Fig. S2: change label “eIF4α” to “eIF4A”
Fig. S7 legend title: delete word “staining”

Reviewer 3

Advance summary and potential significance to field

Kaufman et al., Drosophila sperm development and intercellular cytoplasm sharing through ring canals do not require an intact fusome

The authors use Drosophila as a model to study intercellular bridges (called ring canals [RCs] in Drosophila) connecting germ cells in the testis. They use live imaging to analyze the movement of different proteins through the RCs and show that they are stable and open to selective intercellular traffic throughout spermatogenesis. Intercellular movement through RCs has been extensively characterized by the Cooley lab and others in Drosophila ovaries, in both germ cells and somatic follicle cells, but data on testes was lacking. Here, the authors also look at the role of the fusome, a cytoplasmic structure extending through the RCs, and find that disrupting it has no apparent effect on spermatogenesis or male fertility. This result was unexpected, as previous work from many labs has shown that the fusome plays an important role in oogenesis.

Intercellular bridges between germ cells are an almost universal feature of spermatogenesis throughout the animal kingdom, but their functional significance has not been well characterized in any system, which makes this work novel and relevant to a broad audience.

Comments for the author

Overall, the experiments are clear and carefully controlled and the conclusions are reasonable and well-supported by the data. Only minor changes to the manuscript are needed.

Points to address:

Fig. 3: how many cysts were examined for each GFP-tagged protein? The main conclusion - that a subset of proteins can freely diffuse through the RCs - is well supported by the data but I would hesitate to draw strong conclusions for the proteins that failed to FLIP if only one cyst was examined for each type of protein.

Fig. 5 & discussion (bottom of p. 8): Please clarify what is meant by "abnormal or collapsed" RCs. What criteria were used to distinguish them from normal RCs? The RC shown in panel 5G (inset) does not look as perfectly round as the one in panel 5C. Would it be considered normal or abnormal? Showing a few representative examples of each category could be helpful. Also, in what part of the testis, or in what stage of cyst, were the ring canals that were measured? Do ring canals stay the same size throughout spermatogenesis?

For Spectrin or hts RNAi experiments, please clarify whether the RNAi knockdown took place throughout development or only in adult flies, and state at what temperature the flies were raised to induce expression of the Gal4.

The intriguing finding that hts mutant males are sterile, while germline-specific knockdown has no effect, could be confirmed by knocking down hts only in somatic cells in adult flies.

This is not a critical experiment, but it might be worth mentioning it in the discussion as a future direction.

Please state whether images are single confocal sections or Z-stacks of sections.

Some of the references are incomplete.

First revision

Author response to reviewers' comments

Response to reviews
DEVELOP/2020/190140
Drosophila sperm development and intercellular cytoplasm sharing through ring canals do not require an intact fusome

Responses to reviewer comments are described below and corresponding changes to the text are highlighted in gray in the manuscript. We also made minor revisions (not highlighted) throughout the text to improve readability, clarity, and conciseness.

Reviewer 1

Advance Summary and Potential Significance to Field:
In most organisms, germ cells go through several rounds of incomplete mitosis, forming cysts of cells, which remain interconnected by cytoplasmic bridges. It is often transient in females, whereas it is a well-known feature of spermatogenesis. One hypothesis for its widespread occurrence in males is that sperm needs to share X-linked and Y-linked gene products for their differentiation, while X and Y chromosomes are segregated to different cells during meiosis. In this manuscript, Kaufman and colleagues demonstrate that proteins can move between cells through these cytoplasmic bridges throughout male germ cells development in Drosophila. In addition, they analyzed putative functions of the fusome, which is germline-specific organelle, made of vesicles, and which links cells within each cyst.

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1b) Completeness: my major comment regards the completeness of the analysis of hts mutant phenotypes. The authors convincingly showed that the fusome is absent in hts mutant males, and do not detect any reduction in male fertility. They conclude that disruption of the fusome has no effect on spermatogenesis. However, one major phenotype in hts mutant females is complete cytokinesis during cysts mitosis giving rise to cysts with low and abnormal number of cells, between 2 and 8 cells instead of 16 cells (Yue and Spradlin, 1992). A similar phenotype is found in the absence of a-Spectrin (de Cuevas, et al. 1996). In hts females, most of these abnormal egg chambers lack an oocyte and are not fertile. However, in males, complete cytokinesis in pre-meiotic cells would not necessarily lead to sterility as partial cysts could enter meiosis independently and be fertile. My question is whether the authors have checked cysts integrity in hts mutant males. Are they all made of 16 cells? Have ratios of 2, 4, 8, 16 cells cysts changed? Are cysts divisions still synchronous? I think it is very important to search for these defects, which may not lead to sterility or a reduction in fertility.

We thank the reviewer for this thoughtful comment. In the ovary, lack of a fusome does lead to deleterious effects on oogenesis, however, we have not seen evidence of this in the testis. Using several approaches, we attempted to determine if the number of cells per cyst is perturbed but have found no evidence to support this conclusion. First, we looked at both a cyst membrane marker (FM4-64) and an mCherry-tagged Vasa reporter to define the cysts and count the number of cells within. We found no obvious inconsistencies in cell number in the hts knockdown testis (n=98). Second, we looked at late stage EM micrographs finding no evidence of cysts with fewer than 64 cells in the hts knockdown (n=5). Lastly, in all of our live imaging data, we have not found intact cysts with an incorrect number of cells. Therefore, we conclude that if there is a change in the number of cells per cyst in hts knockdown testes, this effect is subtle and does not seem to affect fertility.

2) Impact:
2a) Novelty/fundamental/broad interest
Movements of proteins between sister-cells in Drosophila males cysts is quite an expected result. It has been shown by the same lab in somatic cells and in female cysts. However, the demonstration is novel in males. These results and conclusions are likely to be conserved in other species, and are thus of broad interest. The absence of defects following the disruption of the fusome is more surprising. However, it needs to be supported by additional experiments (see 1b).
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Minor points: it is not always clear if the authors take into account the GFP tag when they analyzed protein size, as they only analyzed tagged versions of proteins.

To clarify this point, we have added a notation in the Table 1 legend indicating that the protein sizes reflected in the table do not include the size of GFP (pg. 4).

Reviewer 2

Advance Summary and Potential Significance to Field:
Germ cells have long been known to be interconnected by intercellular bridges, or ring canals, that enable clonally related to cells to develop as syncytial cysts. Ring canals have been proposed to permit sharing of cytoplasmic components and signaling molecules needed to coordinate cell growth and differentiation. For example, ovarian ring canals in Drosophila are needed to allow directional transfer of proteins, mRNAs and organelles from nurse cells into the oocyte. In the male germline, ring canals are thought to be important for sharing of post- meiotically transcribed X-linked mRNAs that are needed at later stages of haploid sperm development. However, this has never formally been tested. In Drosophila, a specialized organelle, the fusome, passes through ring canals and is important during oogenesis for coordinating the cell cycle and establishing the identity of the oocyte during germline cyst formation. In Drosophila males, the fusome was recently shown to be important for signaling DNA damage in spermatogonia, leading to cyst elimination. However, the importance of the fusome in developing Drosophila male germ cells has not been rigorously tested.

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Overall, this study is beautifully done, the conclusions are well justified, and the data provide a significant advance in our understanding of the roles of ring canals and the fusome during male

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Comments for the Author:
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We have changed the text to reference the localization of Hts/Adducin to the growing ends of spermatid cysts in both the control and αSpec RNAi genotypes (pg. 5). In reference to controls: "In wild-type testes, the fusome was present throughout all stages of spermatogenesis and extended through RCs connecting all cells within a cyst, and was present at the growing end of spermatid tails (Figure 5A-E)." In reference to αSpec RNAi: "We did, however, observe Adducin cortically at membranes in nos> and bam>αSpec RNAi testes and in elongating spermatid tails (Figure 5G, I, S4H)." Additionally, we have created a new supplementary figure (Figure S7) highlighting Adducin localization at the elongating spermatid tails in the genotypes that gave the most complete fusome knockdown: nos>αSpec and bam>hts knockdown testes. In both genotypes, fusomes were perturbed and Adducin signal localized to the remaining fusome fragments as well as the elongating spermatid tails. From these data and consistent with our previous conclusion, we conclude that an intact fusome is not required for fertility. However, we have softened our claim to account for the observation that remaining Adducin protein, both in fusome fragments and elongating spermatid tails, may be sufficient for fertility (pg. 7). Lastly, we do find that the hts mutants have no Adducin signal at fusomes or elongating spermatid tails suggesting that sterility of that genotype is due to total loss of Hts protein.

2. Along these lines, it would be helpful to provide an image showing the morphology of the clustered Hts/Adducin signals at the growing ends of late-stage elongating spermatid cysts (typically located between Zones 1 and 2) from the testes shown in Fig. 5A-A' and E-E", as it is not clear from the images provided whether the Hts/Adducin signal in α-spectrin knockdowns resembles controls at this stage.

We have included a revised Figure 5 (pg. 6) that includes a new panel showing the localization of Hts/Adducin signal at the growing ends of elongating spermatids (Zone 2). For both wild-type and nos>αSpec RNAi genotypes, the images reflect the region of interest of the highlighted Zone 2 with different maximum Z projections to highlight the spermatid tails. From our images, it is difficult to discern a difference in morphology of the growing ends of late-stage spermatid cysts between αSpec RNAi and control genotypes.*

3. The diagram in Fig. 4T is a bit misleading, as is does not indicate the obvious movement of the PA-GFP away from the ring canals within the spermatids in which PA-GFP has been activated (i.e., in the direction of the arrow pointing towards the "3" in Fig. 4M. Perhaps a skinnier arrow (or the same short arrow as in 4M) could be used to indicate that this also happens, as this movement is quite evident from the corresponding Movie.

Per the reviewer's suggestion, we have added another arrow to the diagram in Figure 4M to indicate movement of PA-GFP in the direction away from the ring canals.

4. It is unclear why the GFP signal in Fig. S2 (1st panel, 2nd row) localizes to ring canals in this image. Is this because Pav-GFP is also being expressed in these cells?

We thank you for bringing this to our attention and have clarified that Pav-GFP is indeed expressed in those cells on the figure.

MINOR CORRECTIONS
p. 3, 10th line from the bottom: delete “and” before “has been implicated”
p. 6, Table 1: change “eIF4AE1” to “eIF4E1”
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Fig. S1 legend: should indicate that GFP and mito-GFP are not FlyTrap lines
Fig. S2: change label “eIF4α” to “eIF4A”
Fig. S7 legend title: delete word “staining”

We thank you for these suggestions and have made all of the corrections in the text. Regarding the addition of an asterisk for Men-B in Table 1, we hope we have made it clear in the text that we did not quantify rate of movement and therefore cannot say with absolute certainty that its movement is indeed more restricted than other proteins.

Reviewer 3

Advance Summary and Potential Significance to Field:
Kaufman et al., Drosophila sperm development and intercellular cytoplasm sharing through ring canals do not require an intact fusome

The authors use Drosophila as a model to study intercellular bridges (called ring canals [RCs] in Drosophila) connecting germ cells in the testis. They use live imaging to analyze the movement of different proteins through the RCs and show that they are stable and open to selective intercellular traffic throughout spermatogenesis. Intercellular movement through RCs has been extensively characterized by the Cooley lab and others in Drosophila ovaries, in both germ cells and somatic follicle cells, but data on testes was lacking. Here, the authors also look at the role of the fusome, a cytoplasmic structure extending through the RCs, and find that disrupting it has no apparent effect on spermatogenesis or male fertility. This result was unexpected, as previous work from many labs has shown that the fusome plays an important role in oogenesis.

Intercellular bridges between germ cells are an almost universal feature of spermatogenesis throughout the animal kingdom, but their functional significance has not been well characterized in any system, which makes this work novel and relevant to a broad audience.

Comments for the Author:
Overall, the experiments are clear and carefully controlled and the conclusions are reasonable and well-supported by the data. Only minor changes to the manuscript are needed.

Points to address:

Fig. 3: how many cysts were examined for each GFP-tagged protein? The main conclusion - that a subset of proteins can freely diffuse through the RCs - is well supported by the data, but I would hesitate to draw strong conclusions for the proteins that failed to FLIP if only one cyst was examined for each type of protein.

We have amended the text to reflect that 2-4 cysts were examined for each genotype (pg. 4).

Fig. 5 & discussion (bottom of p. 8): Please clarify what is meant by "abnormal or collapsed" RCs. What criteria were used to distinguish them from normal RCs? The RC shown in panel 5G (inset) does not look as perfectly round as the one in panel 5C. Would it be considered normal or

abnormal? Showing a few representative examples of each category could be helpful. Also, in what part of the testis, or in what stage of cyst, were the ring canals that were measured? Do ring canals stay the same size throughout spermatogenesis?

We have defined our usage of the word "abnormal" to mean ring canals that are not round and "collapsed" referring to ring canals having no obvious lumen (pg. 6). Additionally, we have highlighted in the text that Figure 5H inset would be an example of an "abnormal" ring canal as it is not as perfectly round as control ring canals. To further illustrate the "abnormal" and "collapsed" ring canal morphologies, we have included an additional supplemental figure (Figure S8) with specific examples and quantification.

Per the reviewer's comment of ring canal measurements, we measured ring canal sizes in spermatogonia and primary spermatocyte cysts. In contrast to female ring canals, male ring canals do not appear to change in size over developmental time.

We would also like to note that we identified a mistake in the number of "abnormal" and "collapsed" ring canals in our initial submission. Previously, the percentage of aberrant ring canals was listed as 8.3% in the text but upon reexamination of the quantified data, we realized the percentage is actually 11.1%. That change has been made in the text (pg. 6) and is reflected in the new supplemental figure in the stacked bar graph (Figure S8C).

For Spectrin or hts RNAi experiments, please clarify whether the RNAi knockdown took place throughout development or only in adult flies, and state at what temperature the flies were raised to induce expression of the Gal4.

We have included the words "throughout development and in adults" to describe the nature of Gal4 expression in the text (pg. 5). Furthermore, we have added a sentence to the methods (pg. 10) detailing the temperatures at which the RNAi experiments were performed: "All animals were raised at room temperature or in a 25°C incubator."

The intriguing finding that hts mutant males are sterile, while germline-specific knockdown has no effect, could be confirmed by knocking down hts only in somatic cells in adult flies. This is not a critical experiment, but it might be worth mentioning it in the discussion as a future direction.

We examined the effect of knockdown of hts specifically in somatic cells by driving hts RNAi with the somatic cell-specific driver c587. We find that overall testis morphology is unaffected and fusomes appear normal when visualized with either aSpectrin or Adducin antibodies. We observe an approximately 50% reduction in fertility, but a comprehensive understanding of this phenotype will be the focus of future work.

Please state whether images are single confocal sections or Z-stacks of sections.

We have amended the methods sections to be clearer in this regard (pg. 10).

Some of the references are incomplete.

We would like to thank the reviewer for bringing this to our attention and have fixed those references that were incomplete.

Second decision letter

MS ID#: DEVELOP/2020/190140

MS TITLE: Drosophila sperm development and intercellular cytoplasm sharing through ring canals do not require an intact fusome

AUTHORS: Ronit S. Kaufman, Kari L. Price, Katelynn M. Mannix, Kathleen M. Ayers, Andrew M. Hudson, and Lynn Cooley
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.

Reviewer 1

Advance summary and potential significance to field

The authors have convincingly answered to my main criticism.

Comments for the author

The authors have convincingly answered to my main criticism.

Reviewer 2

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Germ cells have long been known to be interconnected by intercellular bridges, or ring canals, that enable clonally related to cells to develop as syncytial cysts.
Ring canals have been proposed to permit sharing of cytoplasmic components and signaling molecules needed to coordinate cell growth and differentiation. For example, ovarian ring canals in Drosophila are needed to allow directional transfer of proteins, mRNAs and organelles from nurse cells into the oocyte. In the male germline, ring canals are thought to be important for sharing of post-meiotically transcribed X-linked mRNAs that are needed at later stages of haploid sperm development. However, this has never formally been tested. In Drosophila, a specialized organelle, the fusome, passes through ring canals and is important during oogenesis for coordinating the cell cycle and establishing the identity of the oocyte during germline cyst formation. In Drosophila males, the fusome was recently shown to be important for signaling DNA damage in spermatogonia, leading to cyst elimination. However, the importance of the fusome in developing Drosophila male germ cells has not been rigorously tested.

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Overall, this study is beautifully done, the conclusions are well justified, and the data provide a significant advance in our understanding of the roles of ring canals and the fusome during male germ cell development.

Comments for the author

The manuscript is much improved. The authors have addressed nearly all of my comments and have provided additional data to address gaps in the previous version. I am quite enthusiastic about the current version of the manuscript.
However, I have a few minor comments that I feel should be addressed.

1. The claim that the hts mutant phenotype indicates a role for hts outside the male germline, i.e., in the soma, could be softened further, and the current wording on p. 7 seems somewhat

contradictory. Rather than saying “we posit that the sterility of hrs mutant males may be caused by a fusome-independent function of Adducin. As previously mentioned,”, I suggest replacing “we posit” with “it is possible” and inserting “Alternatively,” before “as previously mentioned,”.

2. As mentioned in my previous review, the Figure S1 legend should be modified to indicate that GFP and mito-GFP are not FlyTrap lines.

3. When describing Figure 6A, A’, the authors refer to “ER-like vesicles frequently embedded in the fusome” (p. 6). It would be helpful if they would indicate these with arrowheads or arrows in Figure 6A or B.

4. The authors describe how they “observe Adducin cortically at membranes” and refer the reader to Figure 5G, I, S4H (p. 5, 2nd line from bottom). Figure S4F also demonstrates this and should be mentioned here.

5. Similarly, the authors describe “Adducin-containing fusome-like fragments” (p. 6, lines 2-3) and refer the reader to Figure 5H and 5J. Figure S4 also shows this and should be mentioned here.

6. Rather than stating, “GFP::Oda did move between cells” (p. 7, 9th line from bottom), it would be less awkward to say “GFP::Oda moved between cells”.

7. The last paragraph of the discussion contains two sentences with redundant wording (p. 9). Rather than saying “through their targeted disruption or occlusion” (final paragraph, lines 2-3) and “perhaps by occlusion or targeted disruption” (final paragraph, line 5), I suggest rewording the first of these sentences to say “however, more could be learned by manipulating RC function.”

Reviewer 3

Advance summary and potential significance to field

please see prior review

Comments for the author

The authors have thoroughly addressed all prior comments (and those from the other reviewers). Publication of this carefully revised manuscript is fully supported.