JCB

mRNA localization mediates maturation of cytoplasmic cilia in Drosophila spermatogenesis

Jaclyn Fingerhut and Yukiko Yamashita

Corresponding Author(s): Yukiko Yamashita, University of Michigan-Ann Arbor

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

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April 24, 2020

Re: JCB manuscript #202003084

Prof. Yukiko M Yamashita
University of Michigan
Life Sciences Institute Room 5403 210 Washtenaw Avenue
Ann Arbor, MI 48109-2216

Dear Prof. Yamashita,

Thank you for submitting your manuscript entitled "Localized mRNA translation mediates maturation of cytoplasmic cilia in Drosophila spermatogenesis". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

You will see that the reviewers found the work of high quality and the results interesting. However, they did not find the claims of local translation supported by the data and asked for clarity on how you view the role of the granules with regards to translation (promoting it or preventing it; Rev#1 #1, Rev#2 #2, #3) and how that fits with dynein incorporation into the axoneme. They additionally had a few other minor points.

We have discussed their remarks and agree that, given the prominence of the translation claims in the manuscript and in your model, translation needs to be experimentally tested for these conclusions to remain. We would welcome consideration of a revision and would leave it to you to decide whether:
- to significantly re-write the text to make it clear local translation is a possible model but is only one of several and removing those claims, while also addressing in full the other issues; or
- to provide new data along the lines of experiments suggested by Reviewers #1 and #2 if you wish to continue to claim there is local translation.

We are prepared to move forward with the paper pending appropriate changes to either substantially alter the local translation claims or provide convincing evidence from a direct experimental test for translation. Please also attend to the referees' other comments and to the points below. We would be happy to discuss the revisions further if this is helpful.

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

1) Text limits: Character count for Articles and Tools 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.

2) 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.
Please note our preferred style and revise accordingly

3) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Please add scale bars to 3F (bottom panels), figure 4 side panels, figure 6 left of I

4) 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: 4GH

5) 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 double-check the methods and provide full descriptions - e.g., for construction of CRISPR lines.
- 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.).

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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.**

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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).

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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.

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.

When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

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 in this letter.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Mark Peifer, PhD
Monitoring Editor, Journal of Cell Biology

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

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

In this new submission, Fingerhut and Yamashita investigate the little understood process of cytoplasmic cilium assembly. Unlike traditional cilia, cytoplasmic cilia have axonemes exposed to the bulk cytoplasm, and they do not rely upon IFT for axoneme elongation, suggesting other mechanisms must be utilized. The authors address how cytoplasmic cilia recruit axonemal dyneins important for axoneme structure and function. Among their findings, depletion of either reptin (rept) or pontin (pont) resulted in loss of outer dynein arm (ODA) and inner dynein arm (IDA) structures from Drosophila spermatocyte cilia.

Building off of their recent work showing axonemal RNAs kl-3 and kl-5 are stored in cytoplasmic granules in spermatocytes, the authors show additional axonemal dynein mRNAs, kl-2 and Dhc98D colocalize within the same structures (the "kl-granules"). The authors investigate the distribution, composition, and regulation of kl-granules and relate these findings to a genetic requirement for male fertility and axoneme structure. They demonstrate the distribution of kl-granules changes during spermiogenesis with granules polarizing to the distal end of early elongating spermatids and then dispersing during later elongation steps. They identify Rept and Pont as colocalizing with and regulating the formation of these granules. Depletion of either rept or pont leads to differential changes in axonemal RNA organization. While all of the RNAs are dispersed during early spermatogenesis stages, showing they play a role in granule assembly, kl-2 and Dhc98D show the normal polarized localization to the distal end of elongating spermatids, suggesting they can bypass the Rept/Pont requirement at this stage. However, these proteins lack classic RNA-binding motifs, so it is unlikely they associate with axonemal RNAs directly. Depletion of rept or pont also resulted in diminished Kl-3 protein levels at the distal end, loss of axoneme association, and reduced levels as detected by western blotting, leading the authors to favor a model where RNA localization coupled to translational control regulates aspects of axoneme maturation.

Overall, the submission provides rich new insights into a little understood process and will be of interest to the field and is appropriate for JCB. The experiments are well conceived and the imaging is nicely presented. However, as currently presented, evidence for local translation is not strong and precisely how the data fit into the proposed model should be clarified.

Major Points:

1. The authors propose local translation of axonemal RNAs provides a dowry of proteins to be used for axoneme incorporation. This conclusion is stated throughout the text in the title. However, evidence for local translation is lacking, and could be toned down. While testing the local translation hypothesis would be welcomed, a key piece of data I felt missing from the submission was a detailed survey of when and where axonemal RNAs and proteins co-existed during spermiogenesis. As the authors have a useful FLAG-KL-3 transgenic animal, it would be helpful to see where KL3 protein resides relative to kl-3 mRNA. This information would clarify precisely when local translation is proposed to occur. Do the authors consider the granules to be permissive or restrictive for translation? Presumably translation does not occur in the spermatocyte granules, because the authors mention they do not see Kl-3 protein forming granules (lines 395-7). This seems like key data and should be mentioned earlier in the text. Does translation occur once the RNAs are dispersed? This would make sense, and would be consistent with the model shown in Fig 7, but is not clearly presented.

2. Related to the above point, have the authors considered the alternate hypothesis that the RNA granules may modulate protein stability? At a minimum, this should be mentioned as a possibility

Minor Points:

1. Neither Rept nor Pont are RNA binding proteins. In previous work (Fingerhut et al 2019), the authors showed that mutation of heph, an RNA binding protein, impaired kl-3 and kl-5 granule formation and led to a reduction in KL-3 protein via western. Given these similar phenotypes, do the authors speculate Heph might function as an adapter between the axonemal RNAs and Rept/Pont? If so, the authors might consider adding this to their discussion and model.

2. On line 243, the authors use saturating imaging conditions to show that rept/pont RNAi led to loss of kl-granules and kl RNAs remained dispersed in the cytoplasm. Based on this, they suggest RNA stability was not affected. This conclusion seems too strong without quantifying smFISH dots. Significantly increasing the LUT of even a control spermatocyte would likely show the same dispersed mRNA signals. The authors show the more convincing data a few lines down via qPCR, so the earlier speculation seems unnecessary.

3. To the list of references provided on line 168, the authors should add the following, which initially reported many of the principles guiding oocyte/embryo germ plasm granule assembly, including suborganization: https://www.ncbi.nlm.nih.gov/pubmed/25848747

4. Suborganization of kl-granules is interesting, and peripheral localization of Dhc98D is convincingly shown in Fig 3D'. It would be helpful if the authors could complement Fig 3F with some form of analysis. A fluorescence intensity profile across a sampling of granules centered at the centroid would be a welcome addition.

5. Related to suborganization, the authors show granule disassembly occurs in a stepwise fashion, with "core" kl-3 and 5 dissociating ahead of the more peripheral kl-2 and Dhc98D mRNAs. This supports the authors' conclusion that the pairs of RNAs seem to be differentially regulated. However, I found this observation a bit counter-intuitive. I would have expected it to be more favorable for the outer RNAs to move off first. Can the authors comment on this?

6. The authors note the interesting finding that kl-2 and Dhc98D mRNAs can bypass the Pont/Rept requirement at later spermiogenesis stages. This might suggest Pont/Rept are more important for these RNAs during earlier developmental steps. The current phrasing on line 257 makes it seem like Rept/Pont are not important and could be modified a bit. Moreover, in spite of this apparent bypass, these animals are still sterile, which is a bit counter-intuitive given the model. Does this suggest that earlier steps of spermatogenesis/granules determine axoneme quality? Or, more simply, the full complement of axonemal RNAs needs to be localized? The authors might consider touching on this idea in the discussion.

7. Related to Major Point 1, did the authors ever try a western of Kl-2 or Dhc98D (do antibodies exist?) following rept or pont RNAi? This could allow one to test if the dispersed RNAs are the translating pool versus the granules and/or further enrich our understanding of the differential regulation of these RNAs relative to the core components.

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

The manuscript by Fingerhut and Yamashita focusses on the maturation process of cytoplasmic axonemes, a very interesting topic that has garnered little attention. This work follows up on a previous observation from the authors that the mRNAs for 2 axonemal dyneins are present in ribonuclear particles (RNPs), or granules, in spermatocytes. Axonemes are known to contain the microtubule motor axonemal dynein that is required for force generation in the flagellum/cilium. Thus a major step in cilia maturation is the addition of dynein component. The authors here have made the discovery that dynein heavy chain mRNAs are localized to granules they term 'kl-granules'. Following meiosis, these granules localize to the distal end of the elongating axoneme near the ciliary cap. Using RNAi analysis and light microscopy, the authors discover that the AAA+ proteins Reptin (Rept) and Potin (Pont) localize to kl-granules, are required for complete kl-granule

assembly, and are required for maintaining proper Kl-3 protein levels. Finally, they show that without Rept or Pont the axonemal dyneins do not load onto axonemes which is the likely cause of infertility.

The localization and phenotypes are very convincing, yet the final model proposed is not supported by the data. As you will see, the authors over-interpret their results to make the conclusion that 1) "local translation" occurs at the distal end of the axoneme and 2) that Rept and Pont control translation as opposed to protein stability.

Main concern

1) Local translation is a well-documented phenomenon in biology, as is RNA granule formation. However, showing that local translation occurs, and investigating the role of mRNA within granules (activation or sequestering) is difficult. In order to claim that local translation occurs, as stated in the title and the second half of the paper, one must show local translation in some way, such as: 1) follow protein production (snap tag technology or other) and the presence of mRNA (ms2, pp7) at the same cytoplasmic locus (preferably live), or at minimum 2) reposition the mRNA through sequestration (knock sideways) and show an effect on protein distribution. The authors do not show these types of experiments to test the local translation hypothesis, thus their title and model are not supported. I suggest that the authors either perform such experiments or, alternatively, qualify ALL statements and their model of local translation using (maybe, might, could), and remove the statement from the title completely. The authors' own sentiment on line 313 "may represent" should be used throughout the entire paper.

It is also not clear why local translation in this case might be beneficial. Why translate at the distal end if maturation occurs at the proximal end? Isn't the idea behind local translation, the local use of protein? Why would it matter where Kl-3 is produced if it's loaded proximally? It is not clear to this reviewer how the authors have demonstrated that distal cytoplasmic localization is required for maturation (as stated on line 368-369). This can only be claimed if the system is manipulated to test such a theory, which leads to the next point.

2) While the authors, have clearly demonstrated that Rept and Pont are required for dynein incorporation into the axoneme (Figure 6), they have not demonstrated that localized translation is required for any step in the process. While this is a reasonable hypothesis consistent with their results, other models could explain these results. For example, the authors state "previous studies have proposed that Rept and Pont function as chaperones in the assembly of axonemal dynein motors". Their results with Rept and Pont could be explained if in the absence of this chaperone activity - dynein protein is produced but fails to assemble into a functional complex competent to incorporate into the axoneme.

They state that they do not favor the hypothesis that Rept and Pont only playing a chaperone role because "no puncta are observed for Kl-3 protein", but I do not see this result. They also argue that the kl-granules are not dynein preassembly complexes because in other systems "dynein preassembly complexes were found to contain proteins (e.g. Wdr78; Huizar et al., 2018) where the Drosophila homolog (Dic61B) mRNAs are not constituents of the kl-granules (Figure S4)." The absence of the dic61B mRNA from kl-granules does not preclude that the Dic61B protein is in these granules and therefore does not support their argument. Furthermore, even if dic61b mRNA tells us where Dic61B protein is localized, Figure S4 shows that the dic61B mRNA is not in the granules in spermatocytes. However, one would predict that dynein assembly complexes would need their complete complement of proteins later in development and the correct experiment would be to look

in spermatids when dynein complexes are assembled and loaded on the axoneme. Taken together, it seems to this reviewer that all their results with Rept and Pont knockdowns could be ascribed to the loss of a chaperone function. Some demonstration of where translation is occurring, and how this position changes without Rept or Pont, would be required to conclude that changed is localized translation is the specific cause of the defects.

Additional concerns

- Have the authors attempted to show kl-3 RNA and Kl-3 protein co-localization in addition to Figure 6A,B?
- Have the authors confirmed that the seminal vesicles are devoid of sperm using DAPI staining for needle nuclei, or by rupturing the vesicles? It is difficult to show the complete loss of sperm in from the phase images.
- Is the sub-granule localization of Reptin the same as Pontin (3F)?
- Lines 195-198: Can the authors speculate why the internal area of a granule might dissociate before the external region? This seems counter intuitive.
- The use of the term 'sub-granule' localization would be helpful in several area such as 256-257.
- The blue in IF images can be hard to see. For the separated channels of images, it is easier to see when they are in gray scale, as the authors do in Figure 4.
- Separated channels of the images in Figure 1E and F are needed.
- In the absence of Rept and Pont, kl-2 and Dhc98D do not form into granules early, but they do in elongating spermatids (Figure 4 I vs. L). Some explanation for how there mRNAs are forming clusters in the distal end of the spermatid hours after elongation has begun is needed. If an experiment such as the one in figure 6 C-H were to be done for the proteins of these dyneins, would they be unaffected by the loss of Pont and Rept?

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

The paper titled, "Localized mRNA translation mediates maturation of cytoplasmic cilia in Drosophila spermatogenesis," is important and clearly demonstrated work from Fingerhut and Yamashita. It finds for the first time that cytoplasmic cilia form by localized translation of axonemal dynein mRNAs in the cytoplasm and that the maturation of cytoplasmic cilia is mediated by the addition of dynein proteins into bare axonemal microtubules directly from the cytoplasm. Cytoplasmic cilia are an example of sperm-specific adaption that is driven by the unique evolutionary selection mechanism that directly works on the sperm, known as postcopulatory sperm competition. This work provides a key insight into the unique mechanism that evolved in drosophila as part of an evolutionary process. This study suggests that re-purposing Reptin and Pontin form a novel structure named kl-granule for this role in sperm cells. This new mechanism provides critical insight into the advantage of cytoplasmic cilia on compartmentalized cilia in long sperm cells. While drosophila sperm are very long, many sperm cells are much longer than most other cell types, and a mechanism of the local translation may be a general mechanism in sperm cells.
I have only minor comments mainly related to quantifying several statements made in the text that is needed to clarify that the observations are regularly sawed.

Minor comments
- "mRNAs within a kl-granule are spatially sub-organized: kl-3 and kl-5 mRNAs,
164 which encode outer dynein arm (ODA) dynein heavy chain proteins, cluster together in the

165 core of the kl-granule while kl-2 and Dhc98D mRNAs, which encode inner dynein arm (IDA)
166 dynein heavy chain proteins, localize peripherally (Fig. 1 E - G)."
Please quantify this localization and test if it is statistically significant.
Fig E and F may benefit from havening a super-resolution image if this is possible.

- Fig 2 - for clarity writhe DAPI in white on a black background so it will mimic the figure

- "such that each resulting haploid spermatid receives a relatively equal amount of kl183 granule (Fig. 2 B)."
Please quantify this statement.

- "Interestingly, some mRNAs dissociate from the kl-granules before others: kl-3 and kl-5
194 mRNAs (encoding ODA proteins) dissociate earlier than kl-2 and Dhc98D mRNAs (encoding
195 IDA proteins) (Fig. 2 D and F).
Please quantify this statement.

- "It is of note that the differential timing of dissociation
196 correlates with the sub-compartmentalization of constituent mRNAs described above: kl-3
197 and kl-5 localize to the core of the kl-granules and dissociate first (Fig. 1 E), whereas kl-2 and
198 Dhc98D localize to the periphery of the kl-granules (Fig. 1 F) and dissociate later.
Please quantify this timing statement.

- "Close examination of
221 the kl-granules in late SCs revealed that Pont is not evenly distributed within a kl-granule
222 and rather concentrates near the core with kl-3 and kl-5 mRNAs (Fig. 1 E and Fig. 3 F). In
223 contrast, kl-2 and Dhc98D mRNAs occupy the periphery of the kl-granule (Fig. 1 F), where
224 Pont is less concentrated."
- Fig 4 E-F Please quantify the dipareses mRNA along the elongating sperm to test if mRNA is enriched at the sperm end even in the absences of the granola.

- "RNAi-mediated knockdown of rept or pont, does not affect IC assembly but does result in disorganized IC progression." - it needs to be calefied in the paper that rept or pont have several mechanisms to cause infertility, and the precise role of the granule cannot be deciphered without blocking them specifically.

- Fig 5 K, like all westerns in the paper, needs to be quantified.

- "ring centriole" I would strongly suggest not to use this term as it is very confusing (it is not a centriole) and call it transition zone.

- IFT was shown to be dispensable for sperm cilium formation also in "Decoding cilia function: defining specialized genes required for compartmentalized cilia biogenesis."

Dear Mark and Melina,

Thank you very much for handling our manuscript "**Localized mRNA translation mediates maturation of cytoplasmic cilia in *Drosophila* spermatogenesis**", which is now changed to "**mRNA localization mediates maturation of cytoplasmic cilia in *Drosophila* spermatogenesis**". We really appreciate all the reviewers for their thoughtful comments and suggestions.

The main issue was that the reviewers felt that our claim of 'localized translation' was not well supported, and suggested that we should 1) edit the text not to claim *localized translation* and/or 2) provide additional experimental data to support the claim. As we discussed during our communication about our revision plan, we modified our text so as not to emphasize 'localized translation', and we also added one supplementary Figure (now Fig. S4) to make the point that Kl-3 protein is indeed concentrated on the tail side within the cyst. As we discussed, the method to directly prove the localized translation of a specific protein (i.e. Kl-3 in this case) is not necessarily straightforward in in vivo systems, and even methods that would indirectly supports our claim would require significant amount of time (to generate antibodies and/or transgenic flies). Considering the fact that the first author is soon to graduate, we thought it is the best if we modify the text and also add available data that favor our claim.

In addition, we have addressed formatting issues/requests: summary statement style, scale bars to all figure panels, requested information for the legend (sample numbers etc), additional method information as requested. Currently, the character count is ~37,700 excluding materials and methods, references, tables, and supplemental legends.

We hope that this revision makes the manuscript acceptable for publication in JCB.

Thank you very much for your help.
Best wishes,
Yukiko

Point-by-point responses
Reviewer comments are in black and our responses are in blue.

Reviewer #1: In this new submission, Fingerhut and Yamashita investigate the little understood process of cytoplasmic cilium assembly. Unlike traditional cilia, cytoplasmic cilia have axonemes exposed to the bulk cytoplasm, and they do not rely upon IFT for axoneme elongation, suggesting other mechanisms must be utilized. The authors address how cytoplasmic cilia recruit axonemal dyneins important for axoneme structure and function. Among their findings, depletion of either reptin (rept) or pontin (pont) resulted in loss of outer dynein arm (ODA) and inner dynein arm (IDA) structures from Drosophila spermatocyte cilia.

Building off of their recent work showing axonemal RNAs kl-3 and kl-5 are stored in cytoplasmic granules in spermatocytes, the authors show additional axonemal dynein mRNAs, kl-2 and Dhc98D colocalize within the same structures (the "kl-granules"). The authors investigate the distribution, composition, and regulation of kl-granules and relate these findings to a genetic requirement for male fertility and axoneme structure. They demonstrate the distribution of kl-granules changes during spermiogenesis with granules polarizing to the distal end of early elongating spermatids and then dispersing during later elongation steps. They identify Rept and Pont as colocalizing with and regulating the formation of these granules. Depletion of either rept or pont leads to differential changes in axonemal RNA organization. While all of the RNAs are dispersed during early

spermatogenesis stages, showing they play a role in granule assembly, kl-2 and Dhc98D show the normal polarized localization to the distal end of elongating spermatids, suggesting they can bypass the Rept/Pont requirement at this stage. However, these proteins lack classic RNA-binding motifs, so it is unlikely they associate with axonemal RNAs directly. Depletion of rept or pont also resulted in diminished Kl-3 protein levels at the distal end, loss of axoneme association, and reduced levels as detected by western blotting, leading the authors to favor a model where RNA localization coupled to translational control regulates aspects of axoneme maturation.

Overall, the submission provides rich new insights into a little understood process and will be of interest to the field and is appropriate for JCB. The experiments are well conceived and the imaging is nicely presented. However, as currently presented, evidence for local translation is not strong and precisely how the data fit into the proposed model should be clarified.

We thank this reviewer for their thoughtful comments and helpful suggestions. We've addressed their comments as follows.

Major Points:

1. The authors propose local translation of axonemal RNAs provides a dowry of proteins to be used for axoneme incorporation. This conclusion is stated throughout the text in the title. However, evidence for local translation is lacking, and could be toned down. While testing the local translation hypothesis would be welcomed, a key piece of data I felt missing from the submission was a detailed survey of when and where axonemal RNAs and proteins co-existed during spermiogenesis. As the authors have a useful FLAG-KL-3 transgenic animal, it would be helpful to see where KL3 protein resides relative to kl-3 mRNA. This information would clarify precisely when local translation is proposed to occur. Do the authors consider the granules to be permissive or restrictive for translation? Presumably translation does not occur in the spermatocyte granules, because the authors mention they do not see Kl-3 protein forming granules (lines 395-7). This seems like key data and should be mentioned earlier in the text. Does translation occur once the RNAs are dispersed? This would make sense, and would be consistent with the model shown in Fig 7, but is not clearly presented.

We agree with this reviewer: while the data are consistent with localized translation, it is just one of several possibilities. Reviewer #2 raised similar points and we've elaborated more about localized translation in response to review #2. Based on our communication with the editors (as mentioned above), we have altered the text to de-emphasize localized translation. Additionally, we appreciate this reviewer's suggestion to perform an IF-RNA FISH experiment to see *kl-3* mRNA and protein side by side. We wish we could do this experiment, but between lab shutdown due to COVID-19 and the impending graduation of the first author, we've instead made an additional supplemental figure (new Figure S4) in which we present stage-matched cells/cysts showing *kl-3* mRNA and protein separately over development. This shows that Kl-3 protein is not present in spermatocytes, the stage where the kl-granules form. There are very low levels of Kl-3 protein in round spermatids, but the signal greatly increases in early-mid elongating spermatids when the kl-granules start to dissociate. This implies that the kl-granules may restrict translation and dissociation might be associated with initiation of translation. Although the granule being a site of translation repression is in line with other cytoplasmic RNP granules, we do not know the exact location of translation (whether it is within the granule or dissociating mRNA). Taking these into consideration, we edited the text to deemphasize 'local translation', while discussing this new data that is in line with (but does not exclusively demonstrate) localized translation.

2. Related to the above point, have the authors considered the alternate hypothesis that the RNA granules may modulate protein stability? At a minimum, this should be mentioned as a possibility

We agree that there are alternative hypotheses. We have amended the text to more thoroughly discuss these possibilities, especially the idea of protein stability (Lines 472-479). In traditional compartmentalized motile cilia assembly, chaperones such as Reptin, Pontin and their associated proteins, have been implicated in axonemal protein stability, although the possibility that they are also functioning in translation has not been tested as axonemal dynein mRNA localization has not been previously observed. Very recently, a preprint (https://doi.org/10.1101/2020.02.26.966754) reported the presence of RNA (using SYTO RNAselect) in Reptin and Pontin containing dynein axonemal particles. As we discuss in our manuscript (lines 477-488), there are similarities and differences between the kl-granules and these particles. Future studies should be aimed at teasing apart these two possibilities for both compartmentalized and cytoplasmic cilia.

Minor Points:

1. Neither Rept nor Pont are RNA binding proteins. In previous work (Fingerhut et al 2019), the authors showed that mutation of heph, an RNA binding protein, impaired kl-3 and kl-5 granule formation and led to a reduction in KL-3 protein via western. Given these similar phenotypes, do the authors speculate Heph might function as an adapter between the axonemal RNAs and Rept/Pont? If so, the authors might consider adding this to their discussion and model.

This is an intriguing possibility. As this reviewer pointed out, neither Reptin nor Pontin are believed to bind RNA. We suspect that Rept/Pont-associated proteins are probably going to have RNA binding capability, but this will be the subject of further study. Additionally, this reviewer is astute in pointing out that there must be a connection between the gene expression program described in our previous work and the kl-granules – how are the kl-granules seeded and how do the mRNAs get to the kl-granules? We discuss in this previous work that Heph may function in RNA processing or export, so theoretically Heph could hand off these mRNAs to kl-granule proteins, but this is very speculative with the data currently collected as Heph does not colocalize with the kl-granules. While beyond the scope of this paper, we're hopeful that a thorough characterization of kl-granule proteins may reveal such an adaptor, and if that is Heph, it would be most exciting.

2. On line 243, the authors use saturating imaging conditions to show that rept/pont RNAi led to loss of kl-granules and kl RNAs remained dispersed in the cytoplasm. Based on this, they suggest RNA stability was not affected. This conclusion seems too strong without quantifying smFISH dots. Significantly increasing the LUT of even a control spermatocyte would likely show the same dispersed mRNA signals. The authors show the more convincing data a few lines down via qPCR, so the earlier speculation seems unnecessary.

We agree that quantifying the smFISH dots would be necessary to make a strong conclusion from this experiment alone, so we have altered the text appropriately to wait until the qPCR experiments are discussed to make a strong conclusion later in the text. We would also add that control images are equally oversaturated so that the number of single dots can be directly visually compared between control and RNAi conditions. Please note that we are 'oversaturation' only the very strong nuclear signal (nascent transcripts), but not to the cytoplasmic signal that is the subject of study here. Combined with the single molecule nature of our FISH, we believe that our method is adequately suited to make the point of mRNA is rather dispersed than being degraded (but this conclusion is better supported later with qPCR results).

3. To the list of references provided on line 168, the authors should add the following, which initially reported many of the principles guiding oocyte/embryo germ plasm granule assembly, including suborganization: https://www.ncbi.nlm.nih.gov/pubmed/25848747

Thank you for bringing this paper to our attention – we have added the citation.

4. Suborganization of kl-granules is interesting, and peripheral localization of Dhc98D is convincingly shown in Fig 3D'. It would be helpful if the authors could complement Fig 3F with some form of analysis. A fluorescence intensity profile across a sampling of granules centered at the centroid would be a welcome addition.

We agree that a complementary analysis would enhance this point, so we have added a fluorescence intensity profile for the image shown in figure 3F.

5. Related to suborganization, the authors show granule disassembly occurs in a stepwise fashion, with "core" kl-3 and 5 dissociating ahead of the more peripheral kl-2 and Dhc98D mRNAs. This supports the authors' conclusion that the pairs of RNAs seem to be differentially regulated. However, I found this observation a bit counter-intuitive. I would have expected it to be more favorable for the outer RNAs to move off first. Can the authors comment on this?

We agree that this is counter-intuitive. We've thought quite a bit about how this may occur, and while it certainly does appear as we describe with *kl-3* and *kl-5* dissociating from *kl-2/Dhc98D*, without direct live observation, we can't be completely sure which mRNAs are dissociating from which, and applying a directionality to this process seems unnecessary at this time. As multiple reviewers had this same reaction, we decided to change the text as we do not wish for this minor point to cause unnecessary confusion. We've therefore altered the text to state that these mRNAs are simply separating from each other. We hope to understand more about the biophysical properties of the kl-granules in the future.

6. The authors note the interesting finding that kl-2 and Dhc98D mRNAs can bypass the Pont/Rept requirement at later spermiogenesis stages. This might suggest Pont/Rept are more important for these RNAs during earlier developmental steps. The current phrasing on line 257 makes it seem like Rept/Pont are not important and could be modified a bit. Moreover, in spite of this apparent bypass, these animals are still sterile, which is a bit counter-intuitive given the model. Does this suggest that earlier steps of spermatogenesis/granules determine axoneme quality? Or, more simply, the full complement of axonemal RNAs needs to be localized? The authors might consider touching on this idea in the discussion.

It is possible that the requirement for Rept/Pont changes over developmental time and may differ between *kl-3/kl-5* and *kl-2/Dhc98D*. We propose on line 257 (now line299-301 and 511-516, after editing) that there may be additional proteins that either work with Rept/Pont or that have a similar function yet localize more specifically to *kl-2/Dhc98D* mRNAs. We have altered the text to make this clearer and address additional possibilities. In regard to the sterility – RNAi of either *kl-3, kl-5, kl-2,* **or** *Dhc98D* results in sterility. Since we have the reagents to fully assess Kl-3, we know that this protein is absent in *rept/pont* RNAi and that alone is sufficient to result in sterility. We wish we had the reagents to properly assess whether Kl-5, Kl-2, and Dhc98D proteins are present following *rept/pont* RNAi, but for now, all we know for sure is that the dynein arms are missing from the axoneme in the EM images presented in figure 6, suggesting that there is also issue with translating/incorporating *kl-2/Dhc98D* despite the ability of these mRNAs to correctly localize in

elongating spermatids in the absence of Rept and Pont. It may be that Rept/Pont associate with a number of other proteins and this proper kl-granule composition could be required for translation.

7. Related to Major Point 1, did the authors ever try a western of Kl-2 or Dhc98D (do antibodies exist?) following rept or pont RNAi? This could allow one to test if the dispersed RNAs are the translating pool versus the granules and/or further enrich our understanding of the differential regulation of these RNAs relative to the core components.

Unfortunately, antibodies do not exist for these proteins, and to our knowledge, no one has created tagged transgenic flies either. We wish we had these sorts of reagents – we'd love to more thoroughly assess Kl-2 and Dhc98D translation/localization.

Reviewer #2: The manuscript by Fingerhut and Yamashita focusses on the maturation process of cytoplasmic axonemes, a very interesting topic that has garnered little attention. This work follows up on a previous observation from the authors that the mRNAs for 2 axonemal dyneins are present in ribonuclear particles (RNPs), or granules, in spermatocytes. Axonemes are known to contain the microtubule motor axonemal dynein that is required for force generation in the flagellum/cilium. Thus a major step in cilia maturation is the addition of dynein component. The authors here have made the discovery that dynein heavy chain mRNAs are localized to granules they term 'kl-granules'. Following meiosis, these granules localize to the distal end of the elongating axoneme near the ciliary cap. Using RNAi analysis and light microscopy, the authors discover that the AAA+ proteins Reptin (Rept) and Potin (Pont) localize to kl-granules, are required for complete kl-granule assembly, and are required for maintaining proper Kl-3 protein levels. Finally, they show that without Rept or Pont the axonemal dyneins do not load onto axonemes which is the likely cause of infertility.

The localization and phenotypes are very convincing, yet the final model proposed is not supported by the data. As you will see, the authors over-interpret their results to make the conclusion that 1) "local translation" occurs at the distal end of the axoneme and 2) that Rept and Pont control translation as opposed to protein stability.

We thank this reader for their thorough assessment and helpful suggestions.

Main concern

1) Local translation is a well-documented phenomenon in biology, as is RNA granule formation. However, showing that local translation occurs, and investigating the role of mRNA within granules (activation or sequestering) is difficult. In order to claim that local translation occurs, as stated in the title and the second half of the paper, one must show local translation in some way, such as: 1) follow protein production (snap tag technology or other) and the presence of mRNA (ms2, pp7) at the same cytoplasmic locus (preferably live), or at minimum 2) reposition the mRNA through sequestration (knock sideways) and show an effect on protein distribution. The authors do not show these types of experiments to test the local translation hypothesis, thus their title and model are not supported. I suggest that the authors either perform such experiments or, alternatively, qualify ALL statements and their model of local translation using (maybe, might, could), and remove the statement from the title completely. The authors' own sentiment on line 313 "may represent" should be used throughout the entire paper.

It is also not clear why local translation in this case might be beneficial. Why translate at the distal end if maturation occurs at the proximal end? Isn't the idea behind local translation, the local use of protein? Why would it matter where Kl-3 is produced if it's loaded proximally? It is not clear to this

reviewer how the authors have demonstrated that distal cytoplasmic localization is required for maturation (as stated on line 368-369). This can only be claimed if the system is manipulated to test such a theory, which leads to the next point.

We fully agree with this reviewer that our evidence for local translation is based on correlation and logical reasoning, but additional experiments such as those proposed by this reviewer would be necessary to directly demonstrate that local translation does in fact occur in this case. Therefore, as described above in response to reviewer #1, we have added a supplemental figure showing stage-matched cells/cysts with kl-3 mRNA and protein expression. While this is not directly addressing local translation, we feel that showing this correlation is important for our proposed model and we realized we did not show Kl-3 protein expression at different stages. Fully and properly showing local translation comes with significant feasibility issues (including generation of new transgenic lines and/or antibodies) and given the current shutdown situation and impending graduation of the first author, we feel that it is not realistic for us to conduct these experiments, especially as many methods would require reagent generation. Additionally, as we do not know how the kl-granule is positioned at the growing end, we cannot re-position it within the cell. Possibly the most feasible experiment would be a proximity ligation assay combined with OP-puro incorporation to show exactly where Kl-3 peptide synthesis occurs within a cell (i.e. colocalization of OP-puro and specific antibody), but these typically do not seem to be combined with mRNA localization. Accordingly, this kind of experiment likely only provides 'consistent' results, but not necessarily 'direct evidence'. Given that the evidence we could provide at this point is not as strong as it can be (under normal circumstances), we have instead gone through the manuscript and edited the text to deemphasize 'localized translation', instead making it one of several likely hypotheses, and added discussion of other possibilities. We also changed the title to 'mRNA localization mediates maturation of cytoplasmic cilia in Drosophila spermatogenesis'.

We also wish to clarify about the notion of cilia maturation that likely led this reviewer to question why localized translation is beneficial. Our data do not support that the maturation occurs at the proximal end. Rather, we believe that the maturation process starts at the distal end. Our model (as depicted in Fig 7) proposes that the distal end of the cilia is maturing while the proximal end is already matured. Accordingly, axonemal proteins are actively translated where mRNAs are, i.e. distal end, and this may facilitate maturation. This is supported by the data presented in Fig.6, where 1) distal region where maturation is happening has high concentration of axonemal proteins, much of them still being in the cytoplasm, and 2) axonemal proteins nicely colocalize with microtubule tracks at the proximal end because maturation is already complete. Failure to properly localize the mRNAs likely prevents this maturation. We have gone through the manuscript to make sure this is more clearly explained.

2) While the authors, have clearly demonstrated that Rept and Pont are required for dynein incorporation into the axoneme (Figure 6), they have not demonstrated that localized translation is required for any step in the process. While this is a reasonable hypothesis consistent with their results, other models could explain these results. For example, the authors state "previous studies have proposed that Rept and Pont function as chaperones in the assembly of axonemal dynein motors". Their results with Rept and Pont could be explained if in the absence of this chaperone activity - dynein protein is produced but fails to assemble into a functional complex competent to incorporate into the axoneme.

They state that they do not favor the hypothesis that Rept and Pont only playing a chaperone role because "no puncta are observed for Kl-3 protein", but I do not see this result. They also argue that the kl-granules are not dynein preassembly complexes because in other systems "dynein

preassembly complexes were found to contain proteins (e.g. Wdr78; Huizar et al., 2018) where the Drosophila homolog (Dic61B) mRNAs are not constituents of the kl-granules (Figure S4)." The absence of the dic61B mRNA from kl-granules does not preclude that the Dic61B protein is in these granules and therefore does not support their argument. Furthermore, even if dic61b mRNA tells us where Dic61B protein is localized, Figure S4 shows that the dic61B mRNA is not in the granules in spermatocytes. However, one would predict that dynein assembly complexes would need their complete complement of proteins later in development and the correct experiment would be to look in spermatids when dynein complexes are assembled and loaded on the axoneme. Taken together, it seems to this reviewer that all their results with Rept and Pont knockdowns could be ascribed to the loss of a chaperone function. Some demonstration of where translation is occurring, and how this position changes without Rept or Pont, would be required to conclude that changed is localized translation is the specific cause of the defects.

Similar to our claims of local translation, we have changed the text to be more inclusive of alternative possibilities as well as more suggestive about the similarities/differences between the kl-granules and dynein preassembly complexes found in other systems. Additionally, we have added the appropriate figure references for lack of Kl-3 protein puncta and switched out the images in Figure S4 (now Figure S5) for early elongating spermatid images for all except for *fzo*, which is utilized at the round spermatid stage for formation of the mitochondrial derivative.

Additional concerns

- Have the authors attempted to show kl-3 RNA and Kl-3 protein co-localization in addition to Figure 6A,B?

As mentioned above, we added a supplemental figure with stage-matched *kl-3* mRNA and protein. We haven't done the combined IF/RNA-FISH and have decided to proceed with the stage-matched images due to the current shutdown situation, as we feel it is clear how the mRNA and protein are localized in relation to each other from these images.

- Have the authors confirmed that the seminal vesicles are devoid of sperm using DAPI staining for needle nuclei, or by rupturing the vesicles? It is difficult to show the complete loss of sperm in from the phase images.

Yes, we've looked at DAPI staining. The phase contrast images are considered the standard for showing seminal vesicles (this is partly because DAPI staining will strongly show nuclei of seminal vesicle epithelia and the lack of needle shaped nuclei is not easy to convey to untrained eyes of general readers) , but for every microscopy experiment (IF, RNA FISH, etc), we looked at the attached seminal vesicles and they were devoid of sperm nuclei in *rept/pont* RNAi conditions.

- Is the sub-granule localization of Reptin the same as Pontin (3F)?

We suspect it would be but we didn't do the IF-RNA FISH experiment with the Reptin antibody because 1) it was a gift from another lab and their stock was low, so we have very little of it and 2) for staining, the Pontin antibody is much more robust. At the least, we know Reptin and Pontin perfectly co-localize with each other with IF.

- Lines 195-198: Can the authors speculate why the internal area of a granule might dissociate before the external region? This seems counter intuitive.

Reviewer 1 has the same comment – we addressed it above in response to their "minor point 5".

- The use of the term 'sub-granule' localization would be helpful in several area such as 256-257.

We agree and have incorporated this term as needed.

- The blue in IF images can be hard to see. For the separated channels of images, it is easier to see when they are in gray scale, as the authors do in Figure 4.

We have gone through the blue split-channel images and made sure that they are adjusted appropriately. We agree that black and white is better for contrast, but with three or four colors, we feel black and white separate channels would get confusing. We chose to use it for figure 4 where we're emphasizing single molecules, which would not be easy to see at all if they were blue.

- Separated channels of the images in Figure 1E and F are needed.

We've added these separated channels.

- In the absence of Rept and Pont, kl-2 and Dhc98D do not form into granules early, but they do in elongating spermatids (Figure 4 I vs. L). Some explanation for how there mRNAs are forming clusters in the distal end of the spermatid hours after elongation has begun is needed. If an experiment such as the one in figure 6 C-H were to be done for the proteins of these dyneins, would they be unaffected by the loss of Pont and Rept?

We have expanded on this point in response to a comment by Reviewer 1 (see their minor point 6). We wish we had antibodies for these proteins in order to do similar experiments as those in figure 6. We suspect that they would be affected by the loss of Rept and Pont as the EM images in figure 6 also showed missing IDAs, which are formed, in part, by Kl-2 and Dhc98D.

Reviewer #3: The paper titled, "Localized mRNA translation mediates maturation of cytoplasmic cilia in Drosophila spermatogenesis," is important and clearly demonstrated work from Fingerhut and Yamashita. It finds for the first time that cytoplasmic cilia form by localized translation of axonemal dynein mRNAs in the cytoplasm and that the maturation of cytoplasmic cilia is mediated by the addition of dynein proteins into bare axonemal microtubules directly from the cytoplasm. Cytoplasmic cilia are an example of sperm-specific adaption that is driven by the unique evolutionary selection mechanism that directly works on the sperm, known as postcopulatory sperm competition. This work provides a key insight into the unique mechanism that evolved in drosophila as part of an evolutionary process. This study suggests that re-purposing Reptin and Pontin form a novel structure named kl-granule for this role in sperm cells. This new mechanism provides critical insight into the advantage of cytoplasmic cilia on compartmentalized cilia in long sperm cells. While drosophila sperm are very long, many sperm cells are much longer than most other cell types, and a mechanism of the local translation may be a general mechanism in sperm cells.
I have only minor comments mainly related to quantifying several statements made in the text that is needed to clarify that the observations are regularly sawed.

We thank this reviewer for their supportive comments.

Minor comments
- "mRNAs within a kl-granule are spatially sub-organized: kl-3 and kl-5 mRNAs, which encode outer dynein arm (ODA) dynein heavy chain proteins, cluster together in the core of the kl-granule while

kl-2 and Dhc98D mRNAs, which encode inner dynein arm (IDA) dynein heavy chain proteins, localize peripherally (Fig. 1 E - G)."
Please quantify this localization and test if it is statistically significant.
Fig E and F may benefit from havening a super-resolution image if this is possible.

We have changed the wording here to say that the mRNAs appear sub-organized instead of that they are sub-organized. As this is minor point of the manuscript, we do not feel that doing any type of colocalization analysis would enhance our message. We have added split channel images for E and F as well as a fluorescence intensity graph so that the localization patterns are clearer. At this time with the lab shutdown, we are unable to switch to super-resolution. For the point we are trying to make, we feel confocal is sufficient.

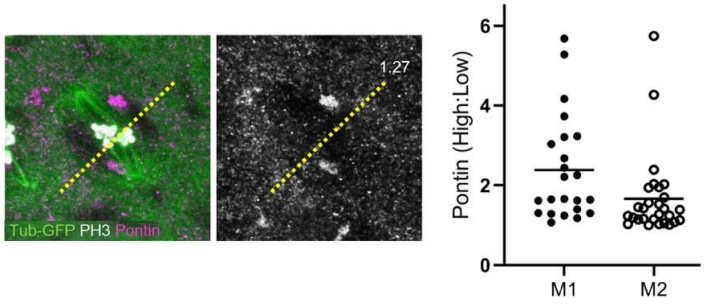
- Fig 2 - for clarity writhe DAPI in white on a black background so it will mimic the figure

We've changed "DAPI" to be white with a black outline so that it more closely resembles the figure.

- "such that each resulting haploid spermatid receives a relatively equal amount of kl granule (Fig. 2 B)."
Please quantify this statement.

We have done this analysis previously (Figure below. M1 is meiosis I, M2 is meiosis II). And most of the case, the ratio of Kl-granule between two daughter cells of meiotic divisions are less than 2-fold (of course there are cells that inherit more/less).

But we would like to note that our statement '**relatively** equal' was not intended to convey any quantitative information (hence 'relatively'). And this statement comes only from a common sense notion that all spermatids would want to inherit some of Kl-granule, but nothing beyond was intended to be conveyed. Because we're not making any claim on the importance of equality of Kl-granule amount, adding this quantitative information (although we have it) is unlikely benefit the manuscript (rather it will be confusing). We hope that this reviewer agrees with this.



- "Interestingly, some mRNAs dissociate from the kl-granules before others: kl-3 and kl-5 mRNAs (encoding ODA proteins) dissociate earlier than kl-2 and Dhc98D mRNAs (encoding IDA proteins) (Fig. 2 D and F).
Please quantify this statement.

As mentioned above, we've reworded this statement to simply say that they are separated as we don't have the time resolution to say which mRNAs are moving in which direction. While we only intended this to be a qualitative statement, we have added a quantification in the text: we observed this

separation in all elongating cysts and have added an n value. However, because this phenomenon was observed in 100% of cysts (n = 269), we are not sure what would be a productive way to further 'quantify' the separation/dissociation.

- "It is of note that the differential timing of dissociation correlates with the sub-compartmentalization of constituent mRNAs described above: kl-3 and kl-5 localize to the core of the kl-granules and dissociate first (Fig. 1 E), whereas kl-2 and Dhc98D localize to the periphery of the kl-granules (Fig. 1 F) and dissociate later.
Please quantify this timing statement.

As mentioned above, we've reworded this statement to simply say that they are separated as we do not have precise timing information.

- "Close examination of the kl-granules in late SCs revealed that Pont is not evenly distributed within a kl-granule and rather concentrates near the core with kl-3 and kl-5 mRNAs (Fig. 1 E and Fig. 3 F). In contrast, kl-2 and Dhc98D mRNAs occupy the periphery of the kl-granule (Fig. 1 F), where Pont is less concentrated."

As it was also pointed out for Figure 1 E and F by this and another reviewer, we've added a fluorescence intensity graph for Figure 3 F to make this point clearer. However, please note that we provided this information as a pure observation (in case this may become important in the future study by us or anybody else), but we do not make any conclusion/statement about the meaning or importance of sub-organization within the granule.

- Fig 4 E-F Please quantify the dipareses mRNA along the elongating sperm to test if mRNA is enriched at the sperm end even in the absences of the granola.

In this case, we feel quantification could cause more confusion than clarity because we do not yet understand completely how the kl-granule mRNAs localize to the distal end of the cyst. We predict that additional proteins, likely associated with Rept/Pont, may be involved, however the degree to which a certain protein localizes a certain RNA is unknown. We therefore don't wish to make any claims as to how dispersed these mRNAs may be (although based on the images, kl-3 and kl-5 mRNAs are greatly mislocalized) as we do not wish to get into these nuances at this time.

- "RNAi-mediated knockdown of rept or pont, does not affect IC assembly but does result in disorganized IC progression." - it needs to be calefied in the paper that rept or pont have several mechanisms to cause infertility, and the precise role of the granule cannot be deciphered without blocking them specifically.

We have clarified this statement within the manuscript. While these axonemal defects/IC cone progression defects for sure result in sterility, whether other functions of rept/pont, if there are other functions in the germ cells, also impact fertility is unknown.

- Fig 5 K, like all westerns in the paper, needs to be quantified.

The expression changes are dramatic and therefore we did not feel the need of quantification back when we conducted western blotting. The same for Rept and Pont protein levels. Although we are aware that many scientists simply use X-ray films for 'quantification' purpose, it is known to be very inaccurate, because the linear range for x-ray film is very limited. When/if we feel the need of quantification, we switch to a phosphorimager to allow accurate quantification, which we did not for

western blotting in this study, because the reduction was obvious. Therefore, although we could provide the imageJ analysis of X-ray film scans, if requested, we know that it is not accurate, and we do not wish to provide seemingly quantitative data, knowing they are not.

- "ring centriole" I would strongly suggest not to use this term as it is very confusing (it is not a centriole) and call it transition zone.

We agree that this term may be confusing as it is not a centriole, however, other literature has made it clear that the ring centriole is separate from the transition zone (see http://dx.doi.org/10.1016/j.cub.2014.09.047) and as Unc-GFP marks the ring centriole, we feel it would be inaccurate to not use this term. We did however change the text to limit our use of "ring centriole" to when we are using Unc-GFP and in other cases we use transition zone.

- IFT was shown to be dispensable for sperm cilium formation also in "Decoding cilia function: defining specialized genes required for compartmentalized cilia biogenesis."

Thank you for pointing this out – we've included this reference.