JCB

Mitochondrial DNA segregation and replication restrict the transmission of detrimental mutation

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July 9, 2019

Re: JCB manuscript #201905160

Dr. Hong Xu
NHLBI, National Institute of Health
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Dear Dr. Xu,

Thank you for submitting your manuscript entitled "Mitochondrial behaviors prime the selective inheritance against harmful mitochondrial DNA mutations". Your manuscript has been assessed by expert reviewers, whose comments are appended below. We sincerely apologize for the delay in communicating our decision to you post-review. Although the reviewers express interest in the work, significant concerns preclude publication of the manuscript in its current form in JCB.

You will see that all the reviewers share the concern that the analyses are not robust and thus not convincing. To address this issue, they suggest straightforward and reasonable experiments that nonetheless represent a significant amount of work. We agree with the reviewers that these additional experiments are needed to bolster your key conclusions. In particular, all of Reviewer #1's comments, which encompass many of the comments from Reviewers #2 and #3 regarding the lack of robustness of the observations, would need to be rigorously addressed for reconsideration at JCB. The Fis1 issue from Reviewer #2 will also need to be addressed using DRP1 KD to strengthen the claims related to mitochondrial fission.

Please let us know if you are able to address the major issues outlined above and wish to submit a revised manuscript to JCB. Our typical timeframe for revisions is three to four months; if submitted within this timeframe, novelty will not be reassessed. We would be open to resubmission at a later date; however, please note that priority and novelty would be reassessed. Thus, if you instead decide to submit elsewhere to potentially publish faster, we understand and can facilitate the transfer of your manuscript and reviews to another journal upon request. For instance, we feel that your manuscript might be appropriate for Molecular Biology of the Cell or Journal of Cell Science. Although we have not discussed your paper with editors at these journals, we could transfer your manuscript files to either journal upon request - please feel free to contact us if you are interested in this option.

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

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

Figures: Your manuscript may have up to 10 main text figures. To avoid delays in production, figures must be prepared according to the policies outlined in our Instructions to Authors, under Data

Presentation, http://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.

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

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

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

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

Sincerely,

Jodi Nunnari, Ph.D.
Editor-in-Chief, Journal of Cell Biology

Melina Casadio, Ph.D.
Senior Scientific Editor, Journal of Cell Biology

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

In this very interesting manuscript, Chen et al looked at the parameters of mtDNA segregation in the germline of Drosophila, addressing the issue of purifying selection. Using a ts mutant that impairs COX activity, they show that its counterselection at NPT is abolished when a COX by-pass using AOX is implemented or if a vital component of the mtDNA replication machinery (mtSSB) or COX itself is downregulated. They also assessed mtDNA copy number, nucleoid number and mitochondrial size at the different stages of oogenesis using various reporter strategies, and performed metabolic labeling to look at mtDNA synthesis. They conclude that a series of events during oogenesis (mitochondrial fragmentation, activation of respiration and a subsequent wave of mtDNA replication) accounts for the preferential elimination of the mutant mtDNA, at the NPT.

On first reading I found that the arguments put forward seemed plausible. However, when I looked closely at the data I noted that the evidence supporting several of these conclusions isn't fully robust. Since the overall question is of such fundamental importance, I feel that the three most important deficiencies do need to be addressed.

1/ All of the assays for the transmission of heteroplasmy that are shown in the manuscript depend on analysing the progeny from single females. Although many such progeny were analysed in each case, the fact that they are all the offspring of just one female of each given genotype means that these are n=1 experiments. To place them on a more reliable footing, at least 4-5 females of each genotype should be looked at in this way. In addition, all of the assays are conducted with the same 'directionality' of temperature shift? Obviously some controls are needed using a shift in the other direction, from NPT rather than to it.

2/ The data from knockdown of mtSSB are interesting, as an indicator that de novo mtDNA replication is required for selection to operate. However, some details are missing, and an important conclusion such as this should not rest on a single RNAi line for a single gene, with no supporting data. The authors should show, as a supplementary file, that this particular RNAi does affect the level of mtSSB RNA in the ovary, when driven by nanos-GAL4 (assuming this was the driver actually used in the experiment). There do not seem to be any other suitable RNAi lines for mtSSB available, although there are VALIUM 20 lines available for tamas, and TFAM which should also be tested. Similar questions arise regarding the UAS-AOX line, details of which were not presented in the earlier MBoC paper; or is the line one of those described by the Kemppainen paper cited there? Was the vector used for constructing the line suitable for germline expression, and was expression in the ovary verified? The data should again be shown in a supplementary file.

3/ More generally, some of the light microscopy images shown are of insufficient resolution to support the stated conclusions. If these images are truly representative, they raise doubts about the numerical findings, especially regarding nucleoid number per mitochondrion and mtDNA copy-number per nucleoid. In particular, all of the ATP synthase subunit α stainings (Fig. 2) look to me too blurry to explain how the authors assigned the outline of individual mitochondria, so as to conduct such counts. The strongly staining ATPα puncta appear to be similar in size and distribution to those visualized by TFAM-GFP, but with a fainter red fluorescence around them (regardless of Fis1 knockdown). What does this mean? Are the red puncta to be considered as mitochondria or as nucleoids or what? More convincing images with better resolution are needed. Similarly, although staining for Vasa allows primordial germ cells to be identified (in Fig. 3A), this is not the same experiment as the FACS-based purification for mtDNA copy number measurement, which used a Vasa-GFP reporter. It also isn't clear to me how green puncta were counted in the Vasa-positive cells in a way that excluded signals from overlaying or adjacent somatic cells. Sample images should be supplied from the other stages for validation.

Minor points

4/ The representative e.m. images are also rather unconvincing. Spheres and tubes can look very similar in simple 2D sections, so I would suggest to implement tomography to get a truer picture of the extent of tubulation in 3D. Even from the numbers in Fig.1B, the effect of Fis1 knockdown does not appear to be dramatic. Moreover, the statistics are questionable: the distribution looks far from normal, so showing a mean and SD - assuming this is what the bar and whiskers denote, which should obviously be stated in the legend - and performing one-way ANOVA, are not appropriate.

5/ Scale bars should be rechecked in all the images. The bars of 10 and 5 μm in Fig.1A and 2A do not seem to me like they can both be correct.

6/ Introduction: The high rate of mtDNA mutation applies only in animals. In plants mtDNA evolves more slowly than nuclear DNA. 'total meltdown' (p. 2, line 9) is too colloquial/metaphorical. The description of the bottleneck is confusing; strictly: the term refers to there being a low number of

heritable units, which can occur either (i) at the organelle (or nucleoid) level during oogenesis, without cell division, as the authors' own work here suggests, or (ii) at the cellular level, by repeated rounds of cell division at relatively low copy number, which is the way the authors generally use the term in this paper. The authors should draw this distinction more clearly and preferably use other terms, such as mitotic segregation. This applies also to the final section of the Results.

7/ Results section 1: The idea of the nucleoid as the unit of inheritance, although proposed by many previous authors, remains unproven. It is also misleading to refer to it as a 'riboprotein' complex, since to most readers this would suggest protein + RNA. Nucleoprotein would be more correct. The repeated reference to 'mitochondrial behaviors' is also a rather strange terminology, since a number of different processes are lumped together under this term (mitochondrial fusion and fission, respiratory activation, DNA replication, aggregation at the fusome, delivery into the oocyte proper). Please find a better way of referring collectively to these processes. 'In consistent' (p. 6, line 25) should be 'Consistent'. What steps did the authors take to ensure that they were looking at mitochondria in germline cysts and not in follicle cells, especially when using light microscopy? The same applies to the EdU labeling shown later in the manuscript (Fig. 6).

8/ Results section 2: the authors record the effect of Fis1 KD on the proportion of progeny mtDNA genotypes after temperature shift. Were other aspects of oogenesis normal?

9/ Results section 3: (p. 9, line 27), TMRM measures membrane potential, not respiration. Membrane potential will also depend on the activity of ATP synthase and other processes that dissipate the proton gradient across the inner membrane. This needs to be better explained/corrected throughout this section.

10/ Results section 4: although mentioned in the Materials and Methods, the authors should state here which GAL4 driver they used to knock down Cox5A or express AOX, to make clear to the reader that these manipulations are germline specific.

11/ Results section 5: the additional selection that is inferred to apply to the Balbiani body is significant but quantitatively very minor. The entire section adds little, and could be dropped.

12/ Discussion: the authors should consider and assess the possible roles in selection of other processes/parameters than respiration, membrane potential and mtDNA replication. These should include ROS, heat production and autophagy (especially in light of the recent paper by Lieber at al). Note also that Fig.1 shows a strong gradient of ATPα expression in the germarium, which may influence these processes.

13/ Figure 5 legend: 'ratiometric', not 'radiometric'.

14/ In many places the authors use the word 'reduced', meaning 'decreased' or 'diminished'. I find this very confusing when used in relation to biological redox processes, and should preferably be replaced with one of the other terms.

15/ Mathematical modeling: I would be more comfortable with this section being checked by a mathematics expert.

16/ Referring back to the Abstract (and Title), I have a number of minor corrections: "few mtDNA repair mechanisms exist" is, for various reasons, incorrect or unproven, as already mentioned above. I think "not all conventional DNA repair systems operate in mitochondria" is more accurate. The

statement: "How the transmission of detrimental mtDNA mutations..." should take a singular verb ("is"). As mentioned above, "mitochondrial behaviours" is too vague a term that is not understandable from previous literature. I'd suggest a re-wording to get around this. In fact, I'm not even sure that behavior can be pluralized like this.

Finally, the 2019 Nature paper by Lieber et al, using different methods, reaches rather similar conclusions about germline selection based on mitochondrial fragmentation followed by programmed fitness-testing (and selective autophagy). These published findings are consistent with the authors' interpretations of their data, but don't obviate the need for them to present fully convincing data of their own, whilst also, to some degree, undercutting the novelty of the current submission.

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

The Chen et al manuscript explores the mechanisms underlying mtDNA selection in the female germline, using Drosophila as a model system. Based on their results, they argue that selection occurs at the organellar level. The number of mtDNA molecules per mitochondrion decreases, allowing the function of the mtDNA molecule to be unmasked. This also occurs around the time that the mitochondria become functionally active, showing increased gene expression and OSPHOS activity.

The authors' models are interesting and reasonable, but the data only moderately support the model. That is, in the absence of stronger data, the model is not compelling. A key part of the model is based on the conclusion that the mtDNA number is decreased to ~1 per mitochondrion in region 2A. I find this conclusion tenuous, based on the images and data presented in Fig. 2. Especially in region 1, the image quality is not good enough to make strong conclusions about number of nucleoids per mitochondrion.

Another key aspect of the model is that fission is important for the selection mechanism. The authors knock down Fis1, a molecule proposed to be involved in fission. However, the role of Fis1 in mammalian mitochondrial fission remains unclear. Their data in Drosophila (Figure 1C) suggests a rather weak/questionable role in fission; there is a substantial overlap in mitochondrial lengths between WT and fis1 knockdowns. It would be less ambiguous to knockdown Drp1, which has a well-recognized role in fission, including in Drosophila. In addition, the effect on selection also seems modest (Fig. 2C). In Fig. 2C, it is also unclear why the fis1 kd shows a different selection number at 18 degrees, compared to controls.

Finally, another key aspect is the role of mitochondrial activity during the selection process. Knockdown of cox5A and mtSSB reduces selection. Their interpretation is that mitochondrial activity differences are measured during the selection process, and that mtDNA replication is important. Another interpretation is that mitochondrial function is important for cell function, and the sick cells caused by cox5A or mtSSB knockdown are simply less efficient at carrying out the selection process (and potentially many other processes). For example, the execution of selection process could be energy dependent.

Other points:

1) In Figure 1, it is more accurate to label the green channel in A as "TFAM" rather than mtDNA.

"mtDNA" is an interpretation.

2) The order of the Figures is unorthodox. The text skips around to different figures, not in the order the figures are presented. For example, much of the data in Fig. 3 is discussed last.

3) The title is ambiguous and could be made more clear.

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

In the manuscript "Mitochondrial behaviors prime the selective inheritance against harmful mitochondrial DNA mutations" by Chen et al, the authors use imaging and sequencing to determine mtDNA replication and mitochondrial morphology during Drosophila oogenesis to test their model replication-competition for selective inheritance (Hill 2014). For this model to work, mtDNA must be whittled down in copy number to a single mitochondrion in order for the mitochondrion and thus the mtDNA to be "tested" for fitness. They use genetics and TFAM GFP to count nucleoid numbers at different points of germ cell development during oogenesis to argue mitochondrial fission is important for purifying selection of a deleterious mtDNA, that there is differential mitochondrial activity corresponding to small, one nucleoid mitochondria compared to longer, multi-copy mitochondria, and there may be a small involvement of mitochondrial transport in the purifying selection. This data provides support for their model, that essentially respiratory function is used as a read out of single mtDNAs to test for fitness.

The authors rely heavily on counting nucleoids using immunofluorescence, but their method is not well described (details below). Furthermore, this manuscript involves developmental timing decisions with respect to germ cell development but there is confusion about what stage they are looking at, and no landmarks to orient and advise their analysis. Furthermore, many experiments involve "knockdown", maybe RNAi?, but this is not adequately described anywhere, nor verified in other ways to ensure the genotype and effect is what they think it is. For these reasons, it is difficult to be confident in their conclusions.

Comments:
abstract
"..mtDNA expression begins..." "The expression of mtDNA allows...". What does this mean exactly? It becomes clearer in the manuscript but is not clear in the abstract.

A fundamental confusion with the data as presented is how the authors are identifying cyst stages in the germarium without markers such as the fusome or ring canals. To complicate this, the authors germarium diagram in Fig. 7 is not consistent with the standard in the literature, making it difficult to understand what region they are referring to, and what stage the cysts are (Mahowald and Strassheim, JCB, 1970). This is true in the figures too. As just one example, in the results section, the authors state (p. 6, line 18) "In the germarium, where the germline stem cells and cystoblasts reside (region 1), ...Mitochondria underwent a notable remodeling in the proliferating cysts (region 2A)....". This is not correct. Region 1 contains GSC, daughter cystoblasts and dividing cysts. Regions 2A/2B contain 16-cell cysts. It is confusing.

Figure 1 recapitulates previously published data that should be acknowledged (p. 6 in results and p. 15 in discussion).
The morphological changes in mitochondria in dividing cysts has previously been shown in fixed and

live tissue (Cox and Spradling, 2003). In addition, a new paper they cite, Lieber et al, has the same finding, but references the previous work. The authors are adding the important data of mtDNA localization, but the observations of morphological changes are not new. Indeed, mitochondria fragment at this stage presumably in response to mitosis (as was also shown), and arguably remain fragmented due to the altered and fast cell cycle at this stage.

Figure 2 - the tfam signal is somewhat clear. There appears to be differences in intensity of the spots. Is this due to copy number of mtDNA, and how are the authors distinguishing different spots from spots that have different intensities for their counts? The materials and methods states "the number of mitochondrial nucleoids per mitochondrion was quantified by manually identifying the nucleoid numbers, indicated as the TFAM-GFP puncta within one mitochondrial fragment". It is a little hard to see the ATPsynthase signal, at least in these representative images. Perhaps the authors can find better examples to support their graph.

It is useful to have counts for the number of nucleoids per mitochondrion in region 2a (Region 1?). Given the size of the fragmented mitochondria in this region, it is not surprising that there is only 1 nucleoid as judged by regular confocal microscopy (not super resolution). They are quite small.

Figure 3. Since the authors are performing their studies from adult ovaries, I would hesitate to draw a firm conclusion of mtDNA copy number present at the end of larval development. The reason is that for adults there is a steady state of egg production that could affect mtDNA copy number, especially since there are physiological differences between adults and previous stages of development. Even though the flies are quite young, they would be starting to ramp up egg production. There should be references for germ cell stage in white pupae in addition to Fig. 3a, there are many available.

For Figure 3B, the comment above about counting tfam spots and their different intensities also applies here. What exactly is being counted? And which stage? Relatedly, is there a difference between 2, 4 and 8 cell cysts? There is no marker for cyst size, such as the fusome or ring canals but perhaps the authors did this. The average number of mitochondria per cell in each stage in the adult ovary is available in a table from Cox & Spradling, 2003. Perhaps this would help the analysis.

The effect of lack of fission should increase the length of mitochondria. For the TEM analysis, how did the authors confirm which stage they were looking at? This can be difficult with sections. ATPsynthase staining may help support this analysis, although the signal in Figure 2 is difficult to see. Also, where exactly is the Bam-GAL4 expressed? Given the confusion on staging, it would be good to have an image with Bam-GAL4 x UASGFP.

In the results section, data presentation skips around in the figures, making it a little difficult to follow. Not sure why the authors chose to do this.

Figure 4. For measuring the intensity of in situ signal, how did the authors know how many cysts were in each image? There should be only one 16-cell cyst in Reg 2B due to the physical constraints of the germarium, but they can be stacked on top of each other in Reg. 2A. It is not indicated in the materials and methods how many sections presumably comprise the Z-stack, nor the number of cysts, either dividing cysts or pre-lens shaped cysts in region 2A. In addition, in region 2B, mitochondria are mostly tightly associated with the fusome and are thus concentrated in one region, presumably concentrating the signal as well (Cox & Spradling, 2003).

Figure 5. The TMRE/Mitotracker Green co-labeling looks convincing with respect to differences

earlier and later in the germarium. Without the fusome, it is hard to say what stage the cysts are. The outlined cysts(s) with higher TMRE labeling look like they could be region 2A/B (thus 16-cell cysts). It is hard to tell.

p. 10, line 5 "Given their co-occurrence, the elevation of respiration is likely due to the onset of mtDNA expression that generates ETCs in 16-cell cysts when selective inheritance begins." Or it could be due to changes in cell cycle regulation and differential metabolic needs.

How was cox5A-KD performed? What was the GAL4 driver used? Is this KD sterile? How was mtDNA heteroplasmy determined (Fig. 5C)? What are the colors in Fig. 5B (if there are two colors, the legend indicates two activities)?

Figure 6. How are the authors distinguishing EdU counts in the germ cells from the surrounding follicle cells? And what is the temperature in panels A and B (it is not indicated in the figure legend). How is the AOX being over-expressed, which GAL4 driver?

The same questions can be asked for the mtSSB knockdown. Are these flies sterile, and if so, how was % heteroplasmy measure, and if not, how effective is the knockdown? What was the GAL4 driver?

Figure 3 How was the milton knockdown done? RNAi? And what is the effect on Balbiani body formation? There is no indication in the materials and methods of the genotype or its effectiveness. It is difficult to draw any conclusions from the heteroplasmy analysis.

p. 12, line 19. "In 16-cell cysts, healthy mitochondria are preferentially transported to the Balbiani body..." I do not believe there is any evidence supporting this.

We would like to thank all reviewers for constructive comments on our manuscript. We have carried out a series of new experiments in response to reviewers' comments and re-written the manuscript accordingly. We believe the revised manuscript is much improved, and hope reviewers will find of our work suitable to be published in *Journal of Cell Biology*. Before going to the detail of point-by-point responses, I would like to first outline the major changes in the revised manuscript.

1. Reviewers concerned that the imaging analysis of mitochondrial fission and nucleoid segregation in *Drosophila* germarium is not robust. In the revision, (A) we carried out focused ion beam scanning electron microscopy (FIB-SEM) and reconstructed 3D volume of *Drosophila* germarium at the ultrastructural resolution. We then used computation segmentation to comprehensively analyze the size and shape of all mitochondria in germarium. We found a sub-population of mitochondria, the elongated, large ones in diving cysts of germarium region 1, become small spheroids in region 2A. Mitochondrial fission is likely involved, as the fragmentation was inhibited in *Fis1 knockdown* germarium. (B) We acquired high-resolution images of mitochondria and mtDNA nucleoids (TFAM-GFP) using stimulated emission depletion (STED) super-resolution microscopy, and re-evaluated the mitochondrial nucleoid segregation process. We found that nucleoids were effectively segregated and sorted into different organelles in region 2A germarium, prior to the onset of selective inheritance. These results are consistent with the conclusions of our previous manuscript.

2. We addressed the comments regarding the knockdown efficiency of the RNAi lines and the fitness of the knockdown flies presented in this study. We also performed additional experiments using *Drp1* and *Tamas* knockdown flies to strengthen our claims that mitochondrial fission and mtDNA replication are required for selective inheritance.

3. To address the concern raised by review#1 regarding the strategy used in the mtDNA selective inheritance assay, we applied a previously published procedure to examine the selective inheritance on more than 10 females for each genotype and replaced all the related data.

4. Both reviewer #2 and #3 complained that the text / data presentation skipped around the figures, and was difficult to flow, specifically related to the quantification of nucleoid numbers in developing germ cells. To improve the overall flow of manuscript, we removed the final section of Results regarding the genetic bottleneck. A recent publication has demonstrated a lack of mitochondrial

genetic bottleneck in *Drosophila* (Hurd et al., 2016). Additionally, as the reviewer #1 pointed out, there seems to be many unsettled issues regarding the mitochondrial bottleneck, which is not the focus of this work at all. As a matter of fact, it was not even mentioned in the abstract of previous manuscript. Thus, removal of the final section of Results will also keep our work more focused.

5. All major revisions on text are highlighted in red.

Below are the point-by-point responses.

Reviewer #1:

In this very interesting manuscript, Chen et al looked at the parameters of mtDNA segregation in the germline of Drosophila, addressing the issue of purifying selection. Using a ts mutant that impairs COX activity, they show that its counterselection at NPT is abolished when a COX by-pass using AOX is implemented or if a vital component of the mtDNA replication machinery (mtSSB) or COX itself is downregulated. They also assessed mtDNA copy number, nucleoid number and mitochondrial size at the different stages of oogenesis using various reporter strategies, and performed metabolic labeling to look at mtDNA synthesis. They conclude that a series of events during oogenesis (mitochondrial fragmentation, activation of respiration and a subsequent wave of mtDNA replication) accounts for the preferential elimination of the mutant mtDNA, at the NPT.

On first reading I found that the arguments put forward seemed plausible. However, when I looked closely at the data I noted that the evidence supporting several of these conclusions isn't fully robust. Since the overall question is of such fundamental importance, I feel that the three most important deficiencies do need to be addressed.

1/ All of the assays for the transmission of heteroplasmy that are shown in the manuscript depend on analysing the progeny from single females. Although many such progeny were analysed in each case, the fact that they are all the offspring of just one female of each given genotype means that these are n=1 experiments. To place them on a more reliable footing, at least 4-5 females of each genotype should be looked at in this way. In addition, all of the assays are conducted with the same 'directionality' of temperature shift? Obviously, some controls are needed using a shift in the other direction, from NPT rather than to it.

We adopted a previously published procedure (Zhang et al., 2019) to examine the selective inheritance in 10 or more females for each genotype. The changes of the *ts* allele from an individual mother to their eggs were quantified upon temperature shift

towards restrictive temperature. We replaced the old graphs in Figure 2E, 3E, 4D and 4E with the new data and revised text accordingly.

Regarding the reviewer's comments on the reverse temperature shifting from NPT to permissive temperature, I would assume that the reviewer is interested in whether the selection is temperature dependent. Previously, both O'Farrell lab (Ma et al., 2014) and my lab (Hill et al., 2014) have independently demonstrated that at permissive temperature, the *ts* allele was stably transmitted over multiple generations in various heteroplasmic lines, indicating a lack of selection. Therefore, we do not think it is necessary to repeat this experiment.

2/ The data from knockdown of mtSSB are interesting, as an indicator that de novo mtDNA replication is required for selection to operate. However, some details are missing, and an important conclusion such as this should not rest on a single RNAi line for a single gene, with no supporting data. The authors should show, as a supplementary file, that this particular RNAi does affect the level of mtSSB RNA in the ovary, when driven by nanos-GAL4 (assuming this was the driver actually used in the experiment). There do not seem to be any other suitable RNAi lines for mtSSB available, although there are VALIUM 20 lines available for tamas, and TFAM which should also be tested. Similar questions arise regarding the UAS-AOX line, details of which were not presented in the earlier MBoC paper; or is the line one of those described by the Kemppainen paper cited there? Was the vector used for constructing the line suitable for germline expression, and was expression in the ovary verified? The data should again be shown in a supplementary file.

We found that *mtSSB* mRNA was reduced in a RNAi background (a TRiP line, BL# 50600) driven by *nanos-gal4*. In knock-down ovaries, mtDNA replication was impaired in germarium region 2B, but largely normal in egg chambers, presumably reflecting that mtDNA replication in region 2B is particularly sensitive to mitochondrial disruption. Female flies produced similar amount of eggs as wild type flies and hatching rate of their eggs was not affected. We included the data of FISH, female fecundity test and egg hatching assay in the Supplementary Figure 2E and Supplementary Figure 4. We also replaced images of EdU incorporation assay with images showing both germaria and egg chambers in the revised Figure 4B.

We also tested RNAi against *tamas* and *tfam*. We found that tamas RNAi also impaired selective inheritance. However, in *tfam* RNAi ovaries, the size of nucleoids was enlarged, while the total number of nucleoids per germ cell was greatly reduced compared to control. This observation is consistent with previous studies showing that knockdown of *tfam* causes enlarged nucleoids (Kasashima et al., 2011), presumably

due to defective mtDNA segregation. Because of these uncharacterized defects in *tfam* knockdown flies, we did not include *tfam* in this study.

The *AOX* transgene was constructed in our lab, using *UASp* vector (Rorth, 1998), which is suitable for expression in *Drosophila* female germline. We clarified this issue in the revised Materials and Methods section on page 18, line 7-9.

3/ More generally, some of the light microscopy images shown are of insufficient resolution to support the stated conclusions. If these images are truly representative, they raise doubts about the numerical findings, especially regarding nucleoid number per mitochondrion and mtDNA copy-number per nucleoid. In particular, all of the ATP synthase subunit α stainings (Fig. 2) look to me too blurry to explain how the authors assigned the outline of individual mitochondria, so as to conduct such counts. The strongly staining ATPα puncta appear to be similar in size and distribution to those visualized by TFAM-GFP, but with a fainter red fluorescence around them (regardless of Fis1 knockdown). What does this mean? Are the red puncta to be considered as mitochondria or as nucleoids or what? More convincing images with better resolution are needed. Similarly, although staining for Vasa allows primordial germ cells to be identified (in Fig. 3A), this is not the same experiment as the FACS-based purification for mtDNA copy number measurement, which used a Vasa-GFP reporter. It also isn't clear to me how green puncta were counted in the Vasa-positive cells in a way that excluded signals from overlaying or adjacent somatic cells. Sample images should be supplied from the other stages for validation.

To improve the image quality and resolution, we carried out stimulated emission depletion (STED) super-resolution microscopy to image mitochondria (ATP synthase) and nucleoids (TFAM-GFP). Acquired image stacks were analyzed in ImageJ. Individual mitochondrion and nucleoid were automatically called out using “color threshold” function and watershed algorithm respectively with manual correction. The detailed criteria and procedure of image analyses were clarified in Materials and Methods and figure legend of Figure 2.

The reviewers’ comment on the ATP synthase staining is spot-on. We were also puzzled by different shapes, intensities of ATP synthase staining, even in the newly acquired STED images. ATP synthase and other respiratory chain complexes have been long thought to uniformly distributed on cristae. However, recent cryo-ET studies showed that ATP synthase forms rows of dimers, and concentrates in the most tightly curved regions along the crista ridges, or around narrow tubular cristae (Davies et al., 2012) (Strauss et al., 2008). It is possible that the different intensities of ATP synthase might be partially due to the different cristae densities in different mitochondria.

We also noticed that TFAM-GFP foci often located in regions that had weaker ATP synthase staining. A recent study using STED nanoscopy demonstrated striking images of mitochondrial cristae and nucleoids at ~50 nm resolution (Stephan et al., 2019). Nucleoids always located in spaces that were devoid of cristae. Thus, the uneven ATP synthase staining within a single mitochondrion could reflects the lack of cristae surrounding nucleoids.

There were about 10% TFAM-GFP foci co-localizing with large, strongly stained ATP synthase puncta, as the reviewer pointed out. We believe these large ATP synthase puncta are likely clusters of multiple mitochondria. Given the limited resolution of optical imaging, we were unable to distinguish each individual organelle, and hence we have excluded this group of TFAM-GFP loci from further analyses.

The confocal images in previous Figure 1 have been replaced with the STED images in revised Figure 2. We also re-wrote related sections in Results, Materials and Methods accordingly.

We include the Vasa-GFP positive and negative cells after FACS in the revised manuscript (Supplementary Figure 3A).

We have deleted the section on the genetic bottleneck in *Drosophila* in the revised manuscript. Therefore, images of mitochondrial nucleoids in germ cells labelled with Vasa staining from the other stages are not relevant anymore.

Minor points

4/ The representative e.m. images are also rather unconvincing. Spheres and tubes can look very similar in simple 2D sections, so I would suggest to implement tomography to get a truer picture of the extent of tubulation in 3D. Even from the numbers in Fig.1B, the effect of Fis1 knockdown does not appear to be dramatic. Moreover, the statistics are questionable: the distribution looks far from normal, so showing a mean and SD - assuming this is what the bar and whiskers denote, which should obviously be stated in the legend - and performing one-way ANOVA, are not appropriate.

Per reviewer's suggestion, we carried out the focused ion beam scanning electron microscopy (FIB-SEM) to reconstruct 3D volume of *Drosophila* germarium at an isotropic resolution of 10x10x10 nm^3 voxels. We then applied the computational segmentation to trace all mitochondria in germ cells. We quantified the relative frequency of mitochondria based on their volume and assessed their geometric shape in region 1 and region 2A of both wild type and *Fis1*-KD germarium. We found that the

abundance of large, elongated mitochondria was decreased in region 2A than region 1, and knock-down of *Fis1* diminished this trend.

We include the FIB-SEM data and related analyses in revised Figure 1, Supplementary Figure 1, Supplementary Table 1 and Supplementary movies.

5/ Scale bars should be rechecked in all the images. The bars of 10 and 5 µm in Fig.1A and 2A do not seem to me like they can both be correct.

We have rechecked and labelled scale bars in all Figures.

6/ Introduction: The high rate of mtDNA mutation applies only in animals. In plants mtDNA evolves more slowly than nuclear DNA. 'total meltdown' (p. 2, line 9) is too colloquial/metaphorical. The description of the bottleneck is confusing; strictly: the term refers to there being a low number of heritable units, which can occur either (i) at the organelle (or nucleoid) level during oogenesis, without cell division, as the authors' own work here suggests, or (ii) at the cellular level, by repeated rounds of cell division at relatively low copy number, which is the way the authors generally use the term in this paper. The authors should draw this distinction more clearly and preferably use other terms, such as mitotic segregation. This applies also to the final section of the Results.

We have revised the text as the reviewer suggested.

Regarding the description of mitochondrial bottleneck inheritance, and how it might occur, we realized there are many unsettled issues. Since it is not the main focus of this study, we decided to remove the final section in the Results. We only touch on this issue in the Introduction and Discussion, to argue for the existence of selection mechanisms on the level of individual organelle / genome, and to introduce our model. We consider the low mtDNA copy number per cell as the mitochondrial bottleneck, which is proposed by experts in mammalian mitochondrial genetics. Given that it is out of our expertise, we chose not to re-define the bottleneck inheritance in this manuscript or engage in any debate on this topic.

7/ Results section 1: The idea of the nucleoid as the unit of inheritance, although proposed by many previous authors, remains unproven. It is also misleading to refer to it as a 'riboprotein' complex, since to most readers this would suggest protein + RNA. Nucleoprotein would be more correct.
The repeated reference to 'mitochondrial behaviors' is also a rather strange terminology, since a number of different processes are lumped together under this term (mitochondrial fusion and fission, respiratory activation, DNA replication, aggregation at

the fusome, delivery into the oocyte proper). Please find a better way of referring collectively to these processes. 'In consistent' (p. 6, line 25) should be 'Consistent'. What steps did the authors take to ensure that they were looking at mitochondria in germline cysts and not in follicle cells, especially when using light microscopy? The same applies to the EdU labeling shown later in the manuscript (Fig. 6).

We deleted the sentence of the “nucleoids are the actual unit of inheritance”. We also replaced the “riboprotein complex” with “nucleoprotein” as the reviewer suggested.

We changed the title to “Developmentally-orchestrated mitochondrial processes prime the selective inheritance against harmful mitochondrial DNA mutations” and replaced “ mitochondrial behaviors” with “mitochondrial processes” in the main text.

In EdU labeling experiment, the ovaries were co-stained with an antibody against fusome structure (Hts-1B1), which allowed us to identify germ cell cysts. The fusome staining are added in Figure 4A and 4B in the revised manuscript.

For other light microscope experiments, in which the antibody staining is infeasible, we considered the position, morphological characters and the nuclear size (Mahowald and Strassheim, 1970), to distinguish germ cells from somatic cells, or to define developmental stages of germ cells. Germarium region 1 mainly contains the big germline stem cell, cystoblast and dividing cysts without follicle cell invasion. Starting from region 2A, the follicle cells start to invade between the germ cell cysts. The clusters are surrounded by only thin strands of prefollicular cells at this stage. Therefore, we focused on the mitochondrial surrounding the big nucleus when analyzing region 2A cysts. The 16-cell cyst in region 2B is flatten and extends the entire width of the germarium. In addition, mitochondria are closely associated with fusome at this region, thus the mitochondrial staining within the cyst at this stage display a long, branched appearance along the fusome structures.

In FIB-SEM images, the size of nuclei and locations of cells were used to distinguish germ cells from somatic cells. For germ cells, their relative locations in a germarium, and the number of interconnected germ cells (judging by presence of ring canals and connecting fusome) within a cyst, were used to determine their developmental stage. The description has been added in figure legend of Figure 1B.

8/ Results section 2: the authors record the effect of Fis1 KD on the proportion of progeny mtDNA genotypes after temperature shift. Were other aspects of oogenesis normal?

The *Fis1* knockdown was driven by *bam-gal4*, which is active in dividing cysts specifically. Overall, the oogenesis of *Fis1* KD appeared normal. The fecundity of the female *Fis* 1 KD fly and the hatching rate of their eggs are comparable to the wild type (Supplemental Figure 4).

9/ Results section 3: (p. 9, line 27), TMRM measures membrane potential, not respiration. Membrane potential will also depend on the activity of ATP synthase and other processes that dissipate the proton gradient across the inner membrane. This needs to be better explained/corrected throughout this section.

We changed “respiration” to “membrane potential” in the Result section (Page 9, line 28-29).

10/ Results section 4: although mentioned in the Materials and Methods, the authors should state here which GAL4 driver they used to knock down Cox5A or express AOX, to make clear to the reader that these manipulations are germline specific.

We specified Gal4 divers in related figure legends and the Materials and Methods section.

11/ Results section 5: the additional selection that is inferred to apply to the Balbiani body is significant but quantitatively very minor. The entire section adds little, and could be dropped.

Balbiani bodies are conserved structures found in developing germ cells from insects to mammals. It has been proposed to play a role in mitochondrial inheritance, but never experimentally tested, at least to our knowledge. Even though its contribution to mtDNA selective inheritance is minor, we believe this issue is worthy to be clarified, and decide to keep this section.

12/ Discussion: the authors should consider and assess the possible roles in selection of other processes/parameters than respiration, membrane potential and mtDNA replication. These should include ROS, heat production and autophagy (especially in light of the recent paper by Lieber at al). Note also that Fig.1 shows a strong gradient of ATPα expression in the germarium, which may influence these processes.

It has been shown that the *ts* allele does not increase ROS production (Chen et al., 2015, Supplemental Figure 3). We have no expertise to assess heat production in insects. Nonetheless, we agree with the reviewer that these two parameters have

important roles in mtDNA selection and evolution, and touch on this aspect in the Discussion (Page 16 Line 28 to Page 17 Line 5).

We also discussed the recent paper by Lieber et al., on page 16, line 2-13 in Discussion.

We think the strong gradient of ATP*α* expression in the germarium is consistent with the notion that mitochondria (respiration) are quiescent in region 1, but become active in region 2. We do not understand how this differential expression pattern of ATP*α* influence ROS, heat-production, or mitophagy.

13/ Figure 5 legend: 'ratiometric', not 'radiometric'.

We have corrected the typo in the revised manuscript.

14/ In many places the authors use the word 'reduced', meaning 'decreased' or 'diminished'. I find this very confusing when used in relation to biological redox processes, and should preferably be replaced with one of the other terms.

We replaced the word “reduced” with “decreased”.

15/ Mathematical modeling: I would be more comfortable with this section being checked by a mathematics expert.

We deleted the final section of Results on genetic bottleneck in revised manuscript. It is a moot point now.

16/ Referring back to the Abstract (and Title), I have a number of minor corrections: "few mtDNA repair mechanisms exist" is, for various reasons, incorrect or unproven, as already mentioned above. I think "not all conventional DNA repair systems operate in mitochondria" is more accurate. The statement: "How the transmission of detrimental mtDNA mutations..." should take a singular verb ("is"). As mentioned above, "mitochondrial behaviours" is too vague a term that is not understandable from previous literature. I'd suggest a re-wording to get around this. In fact, I'm not even sure that behavior can be pluralized like this.

We revised the wording in Abstract per reviewer’s suggestions.

Finally, the 2019 Nature paper by Lieber et al, using different methods, reaches rather similar conclusions about germline selection based on mitochondrial fragmentation

followed by programmed fitness-testing (and selective autophagy). These published findings are consistent with the authors' interpretations of their data, but don't obviate the need for them to present fully convincing data of their own, whilst also, to some degree, undercutting the novelty of the current submission.

We agree that the *Nature* paper by Lieber et al., has partially overlapped findings and conclusions with ours, specifically on the mitochondrial fission part. However, we carried out much more comprehensive and in-depth analyses on mitochondrial morphology (FIB-SEM) and nucleoid segregation (STED microscopy); documented a series of mitochondrial processes including mitochondria dynamics, mtDNA transcription, activation of respiration and mtDNA replication during oocytes development; and examined their contributions to mtDNA selective inheritance. Most importantly, our work supports the model of replication competition, which is completely different from the working model proposed by the *Nature* paper.

The *Nature* paper shows that ATG1 and BINP3 are required for selective inheritance, suggesting that mitophagy is involved. However, previous studies from O'Farrell lab and my lab have demonstrated neither Parkin, the key player of classical mitophagy pathway, nor ATG8 mediated autophagy was involved in selective inheritance. They (Lieber et al.,) proposed that a new type of selective mitophagy is involved. However, it still cannot explain why selective inheritance is completely abolished in *mdi* mutant, which has impaired mtDNA replication in ovaries specifically, but is healthy otherwise. Overall, while there are some overlapped data between these two studies, interpretations are vastly different. We believe it is of great interest to present our work to the field.

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

The Chen et al manuscript explores the mechanisms underlying mtDNA selection in the female germline, using Drosophila as a model system. Based on their results, they argue that selection occurs at the organellar level. The number of mtDNA molecules per mitochondrion decreases, allowing the function of the mtDNA molecule to be unmasked. This also occurs around the time that the mitochondria become functionally active, showing increased gene expression and OSPHOS activity.

The authors' models are interesting and reasonable, but the data only moderately support the model. That is, in the absence of stronger data, the model is not compelling. A key part of the model is based on the conclusion that the mtDNA number is decreased to ~1 per mitochondrion in region 2A. I find this conclusion tenuous, based on the images and data presented in Fig. 2. Especially in region 1, the image quality is

not good enough to make strong conclusions about number of nucleoids per mitochondrion.

Please refer to our responses to point 3 and point 4 of reviewer #1 on this point.

Another key aspect of the model is that fission is important for the selection mechanism. The authors knock down Fis1, a molecule proposed to be involved in fission. However, the role of Fis1 in mammalian mitochondrial fission remains unclear. Their data in Drosophila (Figure 1C) suggests a rather weak/questionable role in fission; there is a substantial overlap in mitochondrial lengths between WT and fis1 knockdowns. It would be less ambiguous to knockdown Drp1, which has a well-recognized role in fission, including in Drosophila. In addition, the effect on selection also seems modest (Fig. 2C). In Fig. 2C, it is also unclear why the fis1 kd shows a different selection number at 18 degrees, compared to controls.

The knockdown was driven by *bam-gal4*, which is active in the dividing cysts, the very stage that the mitochondrial fission was observed (Supplementary Figure 2A). We confirmed that *Fis1* mRNA was indeed decreased in dividing cyst (Supplementary Figure 2B). From FIB-SEM analyses, we found mitochondria encompassed a wide-spectrum of morphology, in term of their shape and size in the germarium. Some mitochondria were small spheres, while others were large, elongated tubules. We found these large, elongated mitochondria in region 1 underwent fission and became smaller spheroids in region 2A. In Fis1 knockdown germarium, the process was compromised (Figure 1, Supplementary Table 1 and Supplementary movies), suggesting that Fis1 promotes mitochondrial fission in *Drosophila*, at least in the early germarium.

Drp1 had higher expression in dividing cysts than other developmental stages of oogenesis based on our FISH data (Supplementary Figure 2C), suggesting that it might be the main regulator of mitochondrial fission in dividing cysts. Thus, we agree with the reviewer that Drp1 might be better choice in this experiment. Regrettably, we were not able to identify an appropriate *Drp1* RNAi line to compromise the mitochondrial fission in region 2A in the initial study. Some *Drp1* RNAi lines including the lines used in Lieber et al., 2019, caused severe ovary degeneration, while others had no effect on mitochondrial morphology. During the revision, we screened all available *Drp1* RNAi lines, and identified one line that impaired the mitochondrial fission in region 2A without disrupting the overall oogenesis. We were unable to carry out FIB-SEM on Drp1 knockdown flies due to the time constrain of the revision. Nonetheless, we confirmed that *Drp1* knockdown also impaired mtDNA selective inheritance and included the data in Supplementary Figure 5A.

The reviewer pointed out there is difference in "*selection number at 18 degrees"* in previous Figure 2C. This reflects the different mutation loads in control and *Fis1* knockdown flies. We replaced this data with new one using another experimental procedure to include more than 10 females for each genotype as reviewer #1 suggested (Figure 2E).

Finally, another key aspect is the role of mitochondrial activity during the selection process. Knockdown of cox5A and mtSSB reduces selection. Their interpretation is that mitochondrial activity differences are measured during the selection process, and that mtDNA replication is important. Another interpretation is that mitochondrial function is important for cell function, and the sick cells caused by cox5A or mtSSB knockdown are simply less efficient at carrying out the selection process (and potentially many other processes). For example, the execution of selection process could be energy dependent.

We agree with the reviewer that knockdown of *cox5A* or *mtSSB* could potentially disrupt overall cellular energy metabolism, and indirectly interferes with the selection process. To address this issue, we overexpressed AOX that can fully rescue homoplasmic *ts* mutant in heteroplasmic flies, and found the selection was greatly diminished. This result indicate that germ cells indeed rely on the respiratory activity of individual mitochondria to gauge the integrity of their mtDNA. We have included the comments in the Results section (Page 10, Line 24-26).

Other points:

1) In Figure 1, it is more accurate to label the green channel in A as "TFAM" rather than mtDNA. "mtDNA" is an interpretation.

We have changed the label in Figure 2 in revised manuscript.

2) The order of the Figures is unorthodox. The text skips around to different figures, not in the order the figures are presented. For example, much of the data in Fig. 3 is discussed last.

To improve the flow of the manuscript, we removed the section on genetic bottleneck, which is not particularly relevant to the main theme of this study anyway.

3) The title is ambiguous and could be made more clear.

We have changed the title to “Developmentally-orchestrated mitochondrial processes prime the selective inheritance against harmful mitochondrial DNA mutations”.

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

In the manuscript "Mitochondrial behaviors prime the selective inheritance against harmful mitochondrial DNA mutations" by Chen et al, the authors use imaging and sequencing to determine mtDNA replication and mitochondrial morphology during Drosophila oogenesis to test their model replication-competition for selective inheritance (Hill 2014). For this model to work, mtDNA must be whittled down in copy number to a single mitochondrion in order for the mitochondrion and thus the mtDNA to be "tested" for fitness. They use genetics and TFAM GFP to count nucleoid numbers at different points of germ cell development during oogenesis to argue mitochondrial fission is important for purifying selection of a deleterious mtDNA, that there is differential mitochondrial activity corresponding to small, one nucleoid mitochondria compared to longer, multi-copy mitochondria, and there may be a small involvement of mitochondrial transport in the purifying selection. This data provides support for their model, that essentially respiratory function is used as a read out of single mtDNAs to test for fitness.

The authors rely heavily on counting nucleoids using immunofluorescence, but their method is not well described (details below). Furthermore, this manuscript involves developmental timing decisions with respect to germ cell development but there is confusion about what stage they are looking at, and no landmarks to orient and advise their analysis. Furthermore, many experiments involve "knockdown", maybe RNAi?, but this is not adequately described anywhere, nor verified in other ways to ensure the genotype and effect is what they think it is. For these reasons, it is difficult to be confident in their conclusions.

Comments:
abstract
"..mtDNA expression begins..." "The expression of mtDNA allows...". What does this mean exactly? It becomes clearer in the manuscript but is not clear in the abstract.

We have revised these sentences in the Abstract.

A fundamental confusion with the data as presented is how the authors are identifying cyst stages in the germarium without markers such as the fusome or ring canals. To complicate this, the authors germarium diagram in Fig. 7 is not consistent with the

standard in the literature, making it difficult to understand what region they are referring to, and what stage the cysts are (Mahowald and Strassheim, JCB, 1970). This is true in the figures too. As just one example, in the results section, the authors state (p. 6, line 18) "In the germarium, where the germline stem cells and cystoblasts reside (region 1), ...Mitochondria underwent a notable remodeling in the proliferating cysts (region 2A)....". This is not correct. Region 1 contains GSC, daughter cystoblasts and dividing cysts. Regions 2A/2B contain 16-cell cysts. It is confusing.

Following reviewer's suggestion, we re-drew the diagram in the revised Figure 6 to make it consistent with the standard in the literature. We also changed the description in the main text to avoid any confusion.

We are grateful to the reviewer for pointing us to the literature on morphological characters of developing cysts. It helped us to better define developmental stages of germ cells in the revision.

Please refer to our response to point 7 of reviewer #1, regarding how to identify the germ cells and their developmental stages in light microscopic and EM images.

Figure 1 recapitulates previously published data that should be acknowledged (p. 6 in results and p. 15 in discussion).
The morphological changes in mitochondria in dividing cysts has previously been shown in fixed and live tissue (Cox and Spradling, 2003). In addition, a new paper they cite, Lieber et al, has the same finding, but references the previous work. The authors are adding the important data of mtDNA localization, but the observations of morphological changes are not new. Indeed, mitochondria fragment at this stage presumably in response to mitosis (as was also shown), and arguably remain fragmented due to the altered and fast cell cycle at this stage.

We referenced previous findings regarding mitochondrial morphological change in revised manuscript.

At this stage, we are not clear the underlying mechanisms of mitochondrial morphological change in early germarium. Certainly, mitotic regulation and fast cell cycle in dividing cyst could be contributing factors.

Figure 2 - the tfam signal is somewhat clear. There appears to be differences in intensity of the spots. Is this due to copy number of mtDNA, and how are the authors distinguishing different spots from spots that have different intensities for their counts? The materials and methods states "the number of mitochondrial nucleoids per

mitochondrion was quantified by manually identifying the nucleoid numbers, indicated as the TFAM-GFP puncta within one mitochondrial fragment". It is a little hard to see the ATP synthase signal, at least in these representative images. Perhaps the authors can find better examples to support their graph.

It is useful to have counts for the number of nucleoids per mitochondrion in region 2a (Region 1?). Given the size of the fragmented mitochondria in this region, it is not surprising that there is only 1 nucleoid as judged by regular confocal microscopy (not super resolution). They are quite small.

In response to the reviewer's concern, we carried out stimulated emission depletion (STED) super-resolution microscopy to visualize mitochondria and nucleoid, and re-evaluated the nucleoids segregation. Please refer to our response to the point 3 of reviewer #1 on this point.

We considered the intensity of TFAM-GFP as a measure for mtDNA copy number within a nucleoid. However, the intensities of TFAM-GFP puncta appeared to be random and continuous, instead of quantized. We think the TFAM-GFP intensity might be influenced by the compaction state of mtDNA, besides the mtDNA copy number in a nucleoid. Thus, the intensity may not be a proper measure for mtDNA copy number.

Figure 3. Since the authors are performing their studies from adult ovaries, I would hesitate to draw a firm conclusion of mtDNA copy number present at the end of larval development. The reason is that for adults there is a steady state of egg production that could affect mtDNA copy number, especially since there are physiological differences between adults and previous stages of development. Even though the flies are quite young, they would be starting to ramp up egg production. There should be references for germ cell stage in white pupae in addition to Fig. 3a, there are many available.

We agree with the reviewer the mtDNA copy number in germ cells at the end of larval development might be different from that in adult germline stem cells. The sorted germ cells from adult ovaries contain germ cells at various developmental stage. To address this problem, we quantified the mtDNA copy number in a female germline stem cell culture (fGS) established from *Drosophila* adult ovaries (Niki et al., 2006). We estimated the number of mtDNA in each fGS cell is about 120, which is similar to the value obtained from germ cells of early pupae ovaries. Still, this is not a perfect experiment, as cultured stem cells are surely different from stem cells *in vivo* to some degree. Nonetheless, the comparable results from two experimental settings strengthen our claims.

We include the data on cultured stem cells in revised Supplementary Figure 3B. We also add reference for germ cell stage in white pupae (Page 7, line 22).

For Figure 3B, the comment above about counting tfam spots and their different intensities also applies here. What exactly is being counted? And which stage? Relatedly, is there a difference between 2, 4 and 8 cell cysts? There is no marker for cyst size, such as the fusome or ring canals but perhaps the authors did this. The average number of mitochondria per cell in each stage in the adult ovary is available in a table from Cox & Spradling, 2003. Perhaps this would help the analysis.

We only counted the number of TFAM-GFP foci, not their intensities. When quantifying nucleoid numbers per mitochondrion, we chose germ cells at the anterior end of region 1, most likely germline stem cells or cystoblasts, and germ cells at region 2A, the 16-cells, to represent the beginning and ending of nucleoid segregation. We did not particularly pay attention to 2,4 and 8 cell cysts.

The effect of lack of fission should increase the length of mitochondria. For the TEM analysis, how did the authors confirm which stage they were looking at? This can be difficult with sections. ATPsynthase staining may help support this analysis, although the signal in Figure 2 is difficult to see. Also, where exactly is the Bam-GAL4 expressed? Given the confusion on staging, it would be good to have an image with Bam-GAL4 x UASGFP.

Please refer to our response to point 4 and point 7 of reviewer #1 regarding FIB-SEM analyses, and how we identified the germ cells and their developmental stages in FIB-SEM images.

Bam-gal4 drive the expression in cystoblasts and dividing cystocytes, and the expression is almost undetectable in 16-cell cyst (Chen and McKearin, 2003). We have included the expression pattern of *bam-gal4* in Supplemental Figure 2A in revised manuscript.

In the results section, data presentation skips around in the figures, making it a little difficult to follow. Not sure why the authors chose to do this.

In order to improve the overall flow and keep the manuscript focused, we deleted the final section regarding mitochondrial bottleneck in the Results.

Figure 4. For measuring the intensity of in situ signal, how did the authors know how many cysts were in each image? There should be only one 16-cell cyst in Reg 2B due

to the physical constraints of the germarium, but they can be stacked on top of each other in Reg. 2A. It is not indicated in the materials and methods how many sections presumably comprise the Z-stack, nor the number of cysts, either dividing cysts or pre-lens shaped cysts in region 2A. In addition, in region 2B, mitochondria are mostly tightly associated with the fusome and are thus concentrated in one region, presumably concentrating the signal as well (Cox & Spradling, 2003).

We appreciate the reviewer's comment. We have re-done the quantification of FISH by normalizing the total florescence intensities to the total volume in different regions of Z stacks spanning the entire depth of germarium. The detailed quantification method is added in the Material and Method section (Page 23, line 1-14).

Figure 5. The TMRE/Mitotracker Green co-labeling looks convincing with respect to differences earlier and later in the germarium. Without the fusome, it is hard to say what stage the cysts are. The outlined cysts(s) with higher TMRE labeling look like they could be region 2A/B (thus 16-cell cysts). It is hard to tell.

The TMRM/MitoTracker Green co-staining assay was conducted in live tissues, and hence we could not co-stain fusome antibody to determine the developmental stages. Instead, we used the morphological characters to distinguish germarium region 2B. The 16-cell cyst in region 2B is flatten and extends the entire width of the germarium. In addition, mitochondria are closely associated with fusome at this region, thus the MitoTracker staining display a long, branched appearance along the fusome structures.

p. 10, line 5 "Given their co-occurrence, the elevation of respiration is likely due to the onset of mtDNA expression that generates ETCs in 16-cell cysts when selective inheritance begins." Or it could be due to changes in cell cycle regulation and differential metabolic needs.

We agree with the reviewer that the elevation of mitochondrial respiration could be responsive to differential metabolic regulation, which may indirectly trigger the expression of ETC subunits encoded on both mtDNA and nuclear genome. The essence of our argument is there will be no mitochondrial respiration without the expression of mtDNA. To clarify on this issue, we change the description to "the elevation of respiration is at least, partially due to the onset of mtDNA expression" on page 10, line 3.

How was cox5A-KD performed? What was the GAL4 driver used? Is this KD sterile? How was mtDNA heteroplasmy determined (Fig. 5C)? What are the colors in Fig. 5B (if there are two colors, the legend indicates two activities)?

Nanos-gal4 (BL#4937) from the Bloomington *Drosophila* Stock Center was used to drive the cox5A RNAi. In fact, strong cox5A RNAi lines driven by *nos-gal4* caused severe ovary degeneration. We identified an appropriate RNAi line that only moderately decreased the mRNA level of *cox5A* (Supplementary Figure 2D) but did not affect the fecundity of the female flies, nor the hatching rate of their progeny (Supplementary Figure 4). Nonetheless, COX activity was markedly decreased in knockdown germarium (Figure 3D). We included the new figures and description in main text (page 10 line 15-20) in the revised manuscript.

In the revised manuscript, we adopted a previously published procedure to examine the selective inheritance on more than 10 females for each genotype (Zhang et al., 2019). The changes of *ts* mtDNA allele between mothers and their eggs were quantified upon temperature shift towards restrictive temperature. The quantification of mtDNA heteroplasmy is described in Material and Methods section.

In the COX/SDH staining assay, the brown color in control indicates both complex II (SDH, blue staining) and complex IV (COX, brown staining) are present and active. Complex IV activity is disrupted by *cox5A* KD in germ cells, while complex II remains intact. Thus, the blue staining in the germarium of *cox5A* KD is derived from the SDH activity. We included the description in figure legend of Figure 3D.

Figure 6. How are the authors distinguishing EdU counts in the germ cells from the surrounding follicle cells? And what is the temperature in panels A and B (it is not indicated in the figure legend). How is the AOX being over-expressed, which GAL4 driver?

In EdU labeling experiment, the ovaries were co-stained with fusome antibody (Hts-1B1), which allowed us to identify germ cells in cysts. The fusome staining are now shown in revised Figure 4A and 4B.

In the figure legend of Figure 4A and 4B, we include the description that the experiment was carried out at 29°C.

We generated UASp-*AOX* transgene and used nanos-gal4 to express it in ovaries. We clarified this issue in the revised Materials and Methods section on page 18, line 7-9.

The same questions can be asked for the mtSSB knockdown. Are these flies sterile, and if so, how was % heteroplasmy measure, and if not, how effective is the knockdown? What was the GAL4 driver?

Please refer to our response to point 2 of reviewer #1 on this point.

Figure 3 How was the milton knockdown done? RNAi? And what is the effect on Balbiani body formation? There is no indication in the materials and methods of the genotype or its effectiveness. It is difficult to draw any conclusions from the heteroplasmy analysis.

Milton was knocked down using a *milton* RNAi line (BL43173) driven by *nanos-gal4* (BL4937) driver. To address the reviewer's concern, we carried out immuno-fluorescence staining in *Drosophila* ovary to examine the formation of Balbiani body. In both forming follicle and stage 5 egg chamber, there are much fewer mitochondria, most of which remained at the anterior end of the oocyte. The phenotypes resemble those of the *milton* null mutant (Cox and Spradling, 2006), indicating the effective disruption of Milton activity and normal Balbiani body formation. The data was included in Figure 5B in revised manuscript.

p. 12, line 19. "In 16-cell cysts, healthy mitochondria are preferentially transported to the Balbiani body..." I do not believe there is any evidence supporting this.

We edited this sentence on Page 12, line 14-17, it now reads as "In germarium region 2B, healthy mitochondria are preferentially associated with fusome (Hill et al., 2014). Some fusome-associated mitochondria will be transported to the Balbiani body and populate mitochondria in the pole plasm (COX and Spradling 2006), the cytoplasm of the future embryo's primordial germ cells (PGCs)"

January 3, 2020

Re: JCB manuscript #201905160R

Dr. Hong Xu
NHLBI, National Institute of Health
10 Center Dr. 10-6C212
Bethesda, MD 20892

Dear Dr. Xu,

Thank you for submitting your revised manuscript entitled "Developmentally-orchestrated mitochondrial processes prime the selective inheritance against harmful mitochondrial DNA mutations". The manuscript has been seen by two of the original reviewers whose full comments are appended below, and we have assessed the revisions requested by the original reviewer #2, who was not available to re-review the manuscript. While the reviewers continue to be overall positive about the work in terms of its suitability for JCB, some outstanding issues remain.

Specifically, reviewer #3 still has concerns that require attention prior to publication. Although reviewer 1 indicated that s/he was satisfied with the revisions, in consultation, s/he agreed that the points raised by reviewer 3 need to be addressed. For the previous points regarding the role of mitochondrial fission/DRP1 in selective mtDNA inheritance raised in the previous round, we appreciate the effort and transparency in pursuing the DRP1 KD fly lines. In the one DRP1 KD fly line that was usable, it is important that you apply the same analysis for mitochondrial size and nucleoid segregation as you did for the Fis1 KD line. As a minor point, please be careful with wording your interpretation of these data as you cannot rule out that mitochondrial fragmentation observed in region 2A is a consequence of increased division or decreased fusion rates. Finally, the reviewers raised the concern of whether the images looked like they have sufficient resolution to accurately identify individual mitochondria to derive the number of nucleoids/mitochondrion. Given this, please ensure that your analysis is sufficiently described such that the readers are able to reproduce the analysis using the published images.

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

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

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

Sincerely,

Jodi Nunnari, Ph.D.

Editor-in-Chief
Journal of Cell Biology

Andrea L. Marat, Ph.D.
Scientific Editor
Journal of Cell Biology

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

The authors have satisfactorily addressed most of the issues I raised earlier.

1/ the method of studying changes in heteroplasmy between generations is now based on multiple females

2/ knockdown at the RNA level is now documented for the mtSSB line, and a tamas RNAi line has been used in addition (please denote this correctly using lower case t in all cases). The FISH data is not fully convincing, although for mtSSB looks a bit better than for some of the other genes, despite the lack of any internal control. Would it not have been possible to do this by qRT-PCR on isolated ovaries? PCR methods should surely be sufficiently sensitive for this.

3/ The light and electron microscopy are considerably improved and the quantitation thus more believable.

_____

I feel that Reviewer 3's criticisms in respect of the points she/he originally raised are valid, and do require attention in a final revision.

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

In the revised manuscript "Developmentally-orchestrated mitochondrial processes prime the selective inheritance against harmful mitochondrial DNA mutations" by Chen et al, the authors use imaging and sequencing to determine mtDNA replication and mitochondrial morphology during Drosophila oogenesis to test their model replication-competition for selective inheritance (Hill 2014). For this model to work, mtDNA must be whittled down in copy number to a single mitochondrion in order for the mitochondrion and thus the mtDNA to be "tested" for fitness. They use genetics and TFAM GFP to count nucleoid numbers at different points of germ cell development during oogenesis to argue mitochondrial fission is important for purifying selection of a deleterious mtDNA, that there is differential mitochondrial activity corresponding to small, one nucleoid mitochondria compared to longer, multi-copy mitochondria, and there may be a small involvement of mitochondrial transport in the purifying selection. Overall, the authors have increased the quality of the images and added further clarification to the text. However, the muddled developmental cyst staging appears to remain from the original version. This confusions seems to carry over into interpretation and gives the impression of overreaching conclusions that are still not well-supported by the images and data collection.

It still remains a little unclear what regions and cyst stage the authors are referring to. While the

authors may have improved Figure 6, the stages are not described well in the figure legend or in the text. This may seem like a small point, however, the conclusions in the manuscript discuss different cyst stages quite a bit emphasizing the importance of Region 2A and whether or not there it mtDNA replication and when mitochondrial morphological changes occur. This appears to be integral to the bam GAL4 driver as well that they use to drive most of the RNAi experiments. It really does lower confidence in the conclusions when the basic developmental biology is not clearly indicated.

For the FIB-SEM analysis, the representative images are very nice however it seems like there may be the intracellular parasite Wolbachia present in the TEM images. Were the authors sure to distinguish between mitochondria and Wolbachia during their analysis? In addition, from the materials and methods, it is not still not quite clear how the authors distinguished between different regions of the germarium making their conclusions about the effect of loss of Fis1 on mitochondrial morphology less certain. Did they base it on cell volume or number of ring canals between germ cells? (based on the Video 1).

For the TFAM/ATP synthase co-labeling in Fig. 2, the pictures are crisper and improved compared to the original manuscript. Unfortunately, although they explain in detail the image manipulation for the analysis, these representative images still make it a little hard to conclude one TFAM spot/mitochondrion and how they would be able to count this. As with the FIB-SEM analysis, did the authors rely on TFAM-GFP to stage the cysts properly? Since there is not an additional established marker, such as the fusome and/or cell membranes it seems hard to know where region 1 and region 2A end and begin.

I may be missing something obvious, but for Figure 3, the authors use smFISH for nucleus- and mitochondria-encoded mRNAs for electron transport complex proteins. They conclude that both sets of mRNAs are upregulated in Region 2B supporting their hypothesis that respiration is initiated at this stage and would contribute to competition between fit and less-fit mitochondrial genomes for replication. mtDNA-encoded signal clearly labels mitochondria. Why should the nucleus-encoded mRNA be labeling mitochondria? Are these two mRNAs (NDUFB5 and COX5A) known to exclusively undergo co-translational import? Or is it antibody labeling to show protein?

2nd Revision - Authors' Response to Reviewers: February 2, 2020

We thank the editor and reviewers for constructive comments on our manuscript. We have carried out a few new experiments/analyses in response to the comments and revised the manuscript accordingly. The changes are:

1. We acquired high-resolution images of mitochondria and mtDNA nucleoids using stimulated emission depletion (STED) super-resolution microscopy in *Drp1* knockdown ovaries (*bam-gal4* driven) (Supplemental Figure 4A &B). We found that it caused similar phenotypes as these in *Fis1* knockdown flies.
2. In the FIB-SEM assay, we added an analysis to compare individual mitochondrial volume among 2-cell, 4-cell and 16-cell cysts (Supplemental Figure 1A). We found mitochondrial volume appeared to be gradually decreased during cyst division. The mitochondria are more fragmented in 16-cell cyst region 2A than dividing cyst in region 1.
3. We did a quantification on the knockdown efficiency of the RNAi lines used in the manuscript (Supplemental Figure 2F)
4. We add more detailed descriptions regarding the programs, scripts, and parameters used for analyzing mitochondria and nucleoid numbers with Image J. We describe the methods used to identify the germ cell stages in FIB-SEM and light microscopic analyses.
5. We revised the wording for description of mitochondrial fragmentation.

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

The authors have satisfactorily addressed most of the issues I raised earlier.

1/ the method of studying changes in heteroplasmy between generations is now based on multiple females

2/ knockdown at the RNA level is now documented for the mtSSB line, and a tamas RNAi line has been used in addition (please denote this correctly using lower case t in all cases). The FISH data is not fully convincing, although for mtSSB looks a bit better than for some of the other genes, despite the lack of any internal control. Would it not have been possible to do this by qRT-PCR on isolated ovaries? PCR methods should surely be sufficiently sensitive for this.

We use lower case "*t*" when describing the "*tamas"* gene in the revised manuscript.

We agree with the reviewer that the efficiency of RNAi knockdown is moderate in most cases. It is consistent with the result that oogenesis progresses normally in these RNAi flies, which has allowed us to assay mtDNA composition in their eggs. RNAi was carried out in germ cells specifically in all experiments. However, there are many other types of somatic tissues in ovaries besides the germ cells. Therefore, qRT-PCR on isolated ovaries would not be effective for evaluating the efficiency of RNAi. Instead, we used FISH fluorescence intensity in follicles cells of each sample as the internal control to evaluate the efficiency of RNAi.

We included the data of FISH quantification in Supplemental Figure 2F and detailed procedures in the section of Materials and Methods.

3/ The light and electron microscopy are considerably improved and the quantitation thus more believable.

I feel that Reviewer 3's criticisms in respect of the points she/he originally raised are valid, and do require attention in a final revision.

Please refer to our responses to reviewer #3.

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

In the revised manuscript "Developmentally-orchestrated mitochondrial processes prime the selective inheritance against harmful mitochondrial DNA mutations" by Chen et al, the authors use imaging and sequencing to determine mtDNA replication and mitochondrial morphology during Drosophila oogenesis to test their model replication-competition for selective inheritance (Hill 2014). For this model to work, mtDNA must be whittled down in copy number to a single mitochondrion in order for the mitochondrion and thus the mtDNA to be "tested" for fitness. They use genetics and TFAM GFP to count nucleoid numbers at different points of germ cell development during oogenesis to argue mitochondrial fission is important for purifying selection of a deleterious mtDNA, that there is differential mitochondrial activity corresponding to small, one nucleoid mitochondria compared to longer, multi-copy mitochondria, and there may be a small involvement of mitochondrial transport in the purifying selection. Overall, the authors have increased the quality of the images and added further clarification to the text. However, the muddled developmental cyst staging appears to remain from the original version. This confusions seems to carry over into interpretation and gives the impression of overreaching conclusions that are still not well-supported by the images and data collection.

It still remains a little unclear what regions and cyst stage the authors are referring to. While the authors may have improved Figure 6, the stages are not described well in the figure legend or in the text. This may seem like a small point, however, the conclusions in the manuscript discuss different cyst stages quite a bit emphasizing the importance of Region 2A and whether or not there it mtDNA replication and when mitochondrial morphological changes occur. This appears to be integral to the bam GAL4 driver as well that they use to drive most of the RNAi experiments. It really does lower confidence in the conclusions when the basic developmental biology is not clearly indicated.

We would like to clarify several key points regarding mitochondrial processes with the corresponding development stages that are described in this study.

Previous studies show that mitochondrial fragmentation takes place in the dividing cysts (Cox and Spradling, 2003). One could deduce that mitochondrial fragmentation is caused by cell division, as suggested by the reviewer in her/his previous comments. However, based on our FIB-SEM data (Supplemental Figure 1A), the fraction of large mitochondria is gradually decreased along the cyst division. It appears that other mechanisms, besides the cell division may also contribute to the mitochondrial fragmentation. After the completion of cyst division, mitochondria become most fragmentated in 16-cell cysts at region 2A. The volume of individual mitochondria in 2-cell, 4-cell and 16-cell cysts were compared in Supplemental Figure 1A in the revised manuscript.

To assay mitochondrial fragmentation and mtDNA segregation, we compared parameters including mitochondrial size, shape and the number of nucleoids per organelle at the beginning (i.e., the most anterior part of region 1) and the end (region 2A), respectively.
Mitochondrial fragmentation takes place in dividing cysts in region 1 and 16-cell cysts in region 2A. We found that RNAi against either *Fis1* or *Drp1* driven by *bam-gal4,* which expresses in the dividing cyst, sufficiently inhibited mitochondrial fragmentation.

Mitochondrial fusion and fission are known to influence mtDNA replication, which begins at the germarium region 2B and plays a major role in the selective inheritance. Therefore, in order not to interfere with mtDNA replication, we did not use *nanos-gal4* that ubiquitously expresses in ovary, to drive RNAi against *Fis1* and *Drp1*.

We have revised manuscripts to clarify the stages of cysts in figure legends and the main text.

For the FIB-SEM analysis, the representative images are very nice however it seems like there may be the intracellular parasite Wolbachia present in the TEM images. Were the authors sure to distinguish between mitochondria and Wolbachia during their analysis? In addition, from the materials and methods, it is not still not quite clear how the authors distinguished between different regions of the germarium making their conclusions about the effect of loss of Fis1 on mitochondrial morphology less certain. Did they base it on cell volume or number of ring canals between germ cells? (based on the Video 1).

We noticed that mitochondria-like objects identified by computational segmentation have different electron density. Majority of objects have dense staining inside, likely the staining on the inner membrane cristae. Another minor population of objects have light staining inside, which could be swollen mitochondria, undifferentiated mitochondria that have less cristae, or *Wolbachia*. A distinct feature of *Wolbachia* is that it is usually encompassed by three layers of membranes, the outermost derived from the host (White et al., 2017). However, we were unable to filter out *Wolbachia* using this feature due to the limited resolution of FIB-SEM images.

We also carried out analyses on the major population of dense-stained objects only, and the trend of mitochondrial fragmentation is essentially the same as shown in Figure 1C and D. Last, but not the least, *Fis1* knockdown, which should have no impact on the morphology of *Wolbachia*, effectively blocked the fragmentation of objects that were segmented, demonstrating that the presence of *Wolbachia* does not change our conclusion. Nonetheless, we revised the Methods and Materials on page 22, line 11-20, to clarify this issue.

To determine the developmental stages of the cysts, we used connecting ring canals to trace all germ cells within a cyst. We revised the Methods and Materials on page 22, line 22-25, to clarify this point.

For the TFAM/ATP synthase co-labeling in Fig. 2, the pictures are crisper and improved compared to the original manuscript. Unfortunately, although they explain in detail the image manipulation for the analysis, these representative images still make it a little hard to conclude one TFAM spot/mitochondrion and how they would be able to count this. As with the FIB-SEM analysis, did the authors rely on TFAM-GFP to stage the cysts properly? Since there is not an additional established marker, such as the fusome and/or cell membranes it seems hard to know where region 1 and region 2A end and begin.

We recognize the limitation of the analyses on nucleoid segregation, which are unavoidable due to the limited resolution of images. We specified programs, scripts and parameters that were applied to analyze images in the Methods and Materials (page 19, line 15 to page 20, line 15), so that reader will be able to reproduce the analysis with the published data.

In FIB-SEM, we used connecting ring canals to trace all germ cells within a cyst, and thereby defined their developmental stages. In immunofluorescence images, we relied on fusome structure, or the position, morphological characters and the nuclear size to define developmental stages of germ cells.

We recognize that it is difficult to define the boundary between region 1 and region 2A. Therefore, to assay nucleoid segregation, we compared the most anterior part of region 1, where stem cells or cystoblasts reside, to region 2A. We have clarified this issue in the main text and figure legends of Figure 2 and Supplemental Figure 4.

I may be missing something obvious, but for Figure 3, the authors use smFISH for nucleus- and mitochondria-encoded mRNAs for electron transport complex proteins. They conclude that both sets of mRNAs are upregulated in Region 2B supporting their hypothesis that respiration is initiated at this stage and would contribute to competition between fit and less-fit mitochondrial genomes for replication. mtDNA-encoded signal clearly labels mitochondria. Why should the nucleus-encoded mRNA be labeling mitochondria? Are these two mRNAs (NDUFB5 and COX5A) known to exclusively undergo co-translational import? Or is it antibody labeling to show protein?

The reviewer did not miss anything. These images are the smFISH, not antibody staining. We did notice that mRNAs of these nuclear-encoded ETC genes appear to co-localize with mitochondria, which is consistent with our previous study showing that all detected nuclear-encoded ETC genes are translated locally on mitochondrial outer membrane (Zhang et al., 2016). In this study, COX5A was detected in the proteomic analysis. It was indeed reduced in *mdi* mutant embryo, suggesting it is translated locally on mitochondrial surface.

We are glad that the reviewer pointed this out. We revised the manuscript on page 9, line 17-20 to emphasize this point.

March 31, 2020

RE: JCB Manuscript #201905160RR

Dr. Hong Xu
NHLBI, National Institute of Health
10 Center Dr. 10-6C212
Bethesda, MD 20892

Dear Dr. Xu,

Thank you for submitting your revised manuscript entitled "Developmentally-orchestrated mitochondrial processes prime the selective inheritance against harmful mitochondrial DNA mutations". Thank you very much for your patience as we and Reviewer #1 assessed the final changes made to the manuscript. We are all supportive of publication and we would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below) and to address the final points made below and by Rev#1. No new experimentation is needed. Most importantly, we hope you and your lab members are well and safe.

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

1) We agree that the text added to address the concerns of Reviewer #3 would benefit from some editing. Also, you still refer to Drp1 as a small, not large, GTPase.

2) Please combine the supplemental figures to meet the limit of 5 supplemental figures. Each figure can span up to one entire page provided that all panels fit on the page.

3) Titles, eTOC: Please consider the following revision suggestions aimed at increasing the accessibility of the work for a broad audience and non-experts.

Title: mitochondria dynamics and replication restrict detrimental mtDNA mutation transmission in the germline
(this is a suggestion, please feel free to edit)

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.

4) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Please add scale bars to 4AB (magnifications)

5) 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: 1CDE, 2CE, 3EB, 4CDE, 5A, S2F, S3B, S4BC, S5-6

6) 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.
- More information about how Dm fecundity and embryo hatch rate were assessed and about mitochondrial activity staining and EdU labelling of Drosophila adult ovaries, and about smFISH even if described in other work.
- Please include the sequences for experimental and control RNAi oligos.
- 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.).

7) A summary paragraph of all supplemental material should appear at the end of the Materials and methods section.
- Please add one brief sentence per item.

8) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

9) Author contributions: A separate author contribution section is required following the Acknowledgments in all research manuscripts. All authors should be mentioned and designated by their full names. We encourage use of the CRediT nomenclature.

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(lhollander@rockefeller.edu).

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

Sincerely,

Jodi Nunnari, Ph.D.
Editor-in-Chief, Journal of Cell Biology

Melina Casadio, Ph.D.
Senior Scientific Editor, Journal of Cell Biology

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

This is a second revision of an earlier manuscript documenting the processes underlying mtDNA segregation and selection in Drosophila oogenesis.

The authors have addressed previous reviewer concerns as follows:

Reviewer 1:

Follicle cell FISH fluorescence intensity has been used as an internal control in the RNAi

experiments (shown in new Fig. S2F). This addresses Reviewer 1's one remaining concern adequately.

Reviewer 3:

Developmental staging and its description in figure legends (6 and S1A): the text, combined with the legend to Fig. 6, seem adequate to describe the proposed behaviour of mitochondria in the developmental sequence. Fig. S1A adds additional data on fragmentation. There remain blemishes in the English/spelling on the relevant figures and legends, as well as scattered throughout the manuscript, which are off-putting, and undoubtedly contributed to the impression of Reviewer 3 that the developmental processes were poorly described. Copy-editing should be checked very carefully at proofing stage by the scientific editor, to ensure that these minor stylistic errors have been fixed correctly but that further errors have not been introduced.

The argument that the authors have excluded any effect of Wolbachia on the findings seems justified, but the relevant data are not actually presented and the description of how this was done seems more appropriate for the Results than for the Materials and Methods section. The text should be rearranged accordingly and the new data should be supplied in a supplementary figure. I would personally have preferred that Wolbachia be more formally excluded using antibiotic treatment, and verification by PCR, followed by a repeat of the entire experiment. However, since Reviewer 3 did not require this, I feel it would not be appropriate to insist on it at this late stage. However, the authors should add a note to the Discussion that the possible presence of Wolbachia as suggested by their TEM images might have influenced the findings, and that it would be appropriate eventually to repeat the experiments using both infected and cured strains to check if there is any effect on mitochondrial behavior. Note that, whilst the authors' approach confines the study of fragmentation to bona fide (densely staining) mitochondria, the entire process of mitochondrial fragmentation and selection could yet be influenced by the presence of the endosymbiont, which is known to have diverse effects on gametogenesis.

Nucleoid number: Reviewer 3 questioned the interpretation of nucleoid number per mitochondrion, based on TFAM/GFP vs. ATP5A signal. The authors have at least provided a comprehensive explanation of their methods and reasoning, although the admitted uneven staining for ATP5A still leaves room for doubt.

Although not clear from the rebuttal letter alone, the fact that ring canals and fusome structure were used to assign developmental stages is included in the manuscript and this is now satisfactory, as is the explanation of how the analysis was cleanly apportioned to region 1 vs. 2A.

The mitochondrial localization of the nuclear-coded mRNAs is now mentioned appropriately in the text.