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Review COMMONS

Comparative analysis of vertebrates reveals that mouse primordial oocytes do not contain a Balbiani body

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Reviewer 1

Evidence, reproducibility and clarity

Laasya Dhandapani, et al have developed elegant methods for comparative characterization based on live imaging of primordial oocytes from Xenopus, mice and humans. Using specific markers for different intracellular organelles and pharmacological antagonists, they show that the three oocytes contain active mitochondria, Golgi apparatus and lysosomes. These organelles are included in a Balbiani body in Xenopus and human but not in mouse oocytes. In the latter they found in place of the Balbiani body a ring-shaped Gologi apparatus and the latter persisted when the oocytes were activated. The authors conclude that mouse oocytes do not contain a Balbiani body and that this structure is not associated with oocyte dormancy contradicting previous reports.
This work is well done and interesting. However the fact that the oocyte of mice did not contain Balbiani body (Title) is not the main result of their work. This has already been shown in other species.
It was shown from a long time that germ cells segregate from the soma early in development and that germ cell fate depends on the acquisition of a germ cell determinant commonly referred to as germplasma, an essential element of germ cell identity. In various animal species the subcellular composition of this germ plasm is different, and it has been shown that it contains various molecules such as RNA, proteins... Oocytes of certain species such as rats, hamsters, guinea pig, rabbits, nonhuman primates and humans have a Balbiani body similar in ultrastructure and behavior to those of Xenopus. But little information on their molecular composition and function is available. Mice oocytes on the contrary do not have any structure corresponding to the germ plasm, and no Balbiani body.
The originality of this work is the comparative analysis of the subcellular composition of the oocytes of these 3 species. The authors show that all three oocytes contain mitochondria, Golgi apparatus, and that all organelles are functional. It was previously postulated that healthy mitochondria selectively associated with the Balbiani body to allow removal of defective mitochondrial genomes from ultimate germ cells.

Significance

The authors show that the ring-shaped Golgi apparatus in mice is not linked to a dormant state of the oocyte. It is known that the Balbiani body or related structure is characteristic of the formation of primordial germ cells which cannot develop when this structure is altered. However the link with a dormant state of the oocyte has not been the main opinion in the literature (see for example the

recent reviews Chun So et al, Trends Cell Biology, 2021,31, 254-267 or Malgorzata Kloc et al, Current topics in Developmental Biology, 2004, 59, 1- 36)..). However the authors do not study the apparition of these structures during development and the possible consequences of blocking different organelles on the subsequent development or function of the oocyte. It was previously hypothesized that the appearance and persistence of a Balbiani body was closely related to the type of the ovary and length of the oogenesis, and appears when oogenesis is rather slow but not in rapid oogenesis because the formation and transport of germplasm component occur rapidly (above References).

It is therefore an important elegant and detailed live-imaging study of germplasm composition in Xenopus, mice and humans which provides a true original knowledge. It should be noted that oocytes were isolated from neonatal mice, young adult Xenopus and from women aged 18 to 37 years. Further imaging and functional studies are needed to understand the function of the Balbiani body or the ring-shaped Golgi apparatus in germ cell growth, differentiation polarity and fertilization.

Reviewer 2

Evidence, reproducibility and clarity

Asymmetric localization of cellular components is a shared feature of early oocytes. The Balbiani body is a nonmembrane bound compartment that has been observed in species ranging from insects to vertebrates, including mammals. This structure has been postulated to play a role in regulating oocyte dormancy, RNA localization and regulation, and sequestration of determinants, such as the germ plasm and patterning molecules in some animals, and allocation or sorting of cellular organelles, namely mitochondria. In recent years, the Balbiani body has been shown to assemble by a mechanism that involves intrinsically disordered proteins and amyloid formation. In this work the primary oocytes of three species, Xenopus, mouse, and human are characterized using live imaging, vital dyes, and inhibitors to probe the distribution and activity of cellular organelles. The live imaging approach that sets this work apart from previous studies examining cellular asymmetries in mouse, Xenopus, and human oocytes. Interestingly, this study revealed that lysosomes are active in vertebrate oocytes. In contrast, lysosomes are not thought to be active in the invertebrates examined thus far. The manuscript is well written and has several new and exciting findings, including a more detailed comparison of asymmetries and organelles within vertebrate oocytes that challenges the existing models for mammalian Balbiani body composition that were based largely on the mouse oocyte. Overall, the experiments appear to be well controlled, the data are of high quality, and the data provided largely support the conclusions, but could be strengthened by attending to some easily addressable matters, center around missing or unclear numbers for some of the experiments and that some of the analysis appears to have only been quantified in the mouse. Given that this is a comparative study, and that a key conclusion is that the mouse is different, the supporting quantitative data should be provided.

Major:
One potential concern is that the human oocytes used were obtained from women who had undergone ovarian surgery. If the women had surgery because of cysts, cancer, or similar reproductive health concerns, then it is possible that the oocytes may not represent are healthy normal oocytes. If this is a possibility, then this should be stated. It is probably a good idea to clarify even if it is not a concern - if these were all or in part from healthy women free of disease affecting the reproductive system.

In Figure 1 panel C quantification of lysotracker is shown for mouse oocytes. Was similar analysis performed on the lysotracker labeled Xenopus and human oocytes? Including this analysis would provide a quantitative basis for comparison of these different oocytes.

Based on the TMRE staining shown in Figure 1H, the mouse and human oocytes appear similar. Both appear to have domains devoid of mitochondria? For the Xenopus and human oocyte analyses, how many oocytes were examined? From how many females? It would be helpful to include the specific quantification and criteria that were used to conclude that mitochondria distribution is different in human and mouse oocytes- Based on the examples shown, human looks quite different from

Xenopus and more similar to the mouse oocyte. Is this a function of stage? The schematics in panel I are helpful, but please add labels above to indicate the species.

It is not clear how many oocytes and individuals were examined for the proteostat experiments.

Minor:

A general comment for the graphs in figures where oocytes of more than one species are shown, please include labels indicating which species is shown. If a graph or schematic contains data from more than one species, please add a key.

The title for Figure 2 concludes that mitochondria of mouse primordial oocytes are not maintained within a Balbiani body, but mitochondria are not analyzed in the figure. Please consider revising to reflect the data shown or include data on mitochondria.

Scale bars are missing in Figure 3A, for the enlarged panels and insets in Figure 4, Supp. Fig 1A top row, and Supp. 4A

In Supp. Fig 1C and Supp. Fig. 2 - assume the samples shown are mouse but this is not indicated.

Supp. Fig. 2C - please show each sample point sas in the other figures.

Significance

Previous analysis of mouse primary oocytes identified a Golgi ring as the mouse Balbiani body. Based on the lack of mitochondria accumulation in this structure, it was suggested that the mammalian Balbiani body was unique from those of nonmammalian vertebrates. A striking finding from this work is that the mouse indeed appears to be unique but that somewhat unexpectedly, the primary oocytes of Xenopus and human appear to share similar cellular structures. Specifically, the distribution of organelles is different in mouse oocytes, and they appear to lack Balbiani bodies based on several criteria examined in this study: 1) no accumulation of mitochondria, 2) no evidence for RNA localization within a nonmembrane bound compartment, and 3) no evidence for an amyloid-like matrix. Moreover, staining and inhibitor treatments indicate that the Golgi ring has the hallmarks of a conventional Golgi structure and has no correlation to or instructive role in preventing or promoting oocyte activation, but does disassemble in response to oocyte activation. This work challenges the notion that mice even have a Balbiani body and provides evidence against the prevailing model that the Golgi ring regulates oocyte dormancy. The findings point to the importance of choosing a system to model a process based on a comparative cell biology approach rather than solely on evolutionary distance. The work is well-done, and the findings are exciting and significant to reproductive biologists investigating vertebrate oocyte biology, such as myself, and more broadly to cell biologists, developmental biologists, geneticists, and those investigating fertility and reproductive health.

Reviewer 3

Evidence, reproducibility and clarity

In this manuscript the authors aim to compare the content and organization of the Balbiani body between Xenopus, mouse and human oocytes. The Balbiani body is a conserved feature of oocytes across the animal kingdom. In frog and fish the Balbiani body is essential for oocyte polarity, proper fertilization and inheritance of maternal regulators that induces the dorsal-ventral axis of the future embryo, as well as its germline. In mouse, the Balbiani body has been associated with culling of successful oocytes during early formation of the primordial and primary follicle. Understanding the formation, organization and various features of the Balbiani body is therefore of great importance. While the Balbiani body has been extensively functionally studied in frog and fish, this aim is very important in mammals where functional tools for Balbiani body investigation are not yet available.

Based on vital dye labeling the authors examine the organization of the Balbiani body and conclude that mouse oocytes do not contain a Balbiani body. Unfortunately, I have substantial concerns regarding the experiments shown and I could not find the data compelling to support such a conclusion. Generally, the authors heavily rely on the use of vital dyes and do not employ complementing methodologies or approaches to support these findings. The rational of some of the experiments is not clear and rely on weak assumptions and some controls are missing. In addition, the number of oocytes/ovaries examined is rarely provided, and the presentation of the data is not always clear. I am detailing my comments below.

The authors conclude the "mouse oocytes do not contain a Balbiani body". As a constructive advice, I would like to comment that it is extremely challenging conceptually to prove that something does not exist. It may very well be that the mouse Balbiani body is different than that of other species in its content and/or organization, and I believe that the authors should consider this possibility.

Major Comments
Fig. 1A - LysoTracker signal in the Xenopus oocyte looks more like background than a true signal. Also, the insert hides much of the shown oocyte, it is advisable to show the entire oocyte. Based on the images shown it is difficult to conclude that "vertebrate primordial oocytes have acidic, active lysosomes distributed in their cytoplasm" for the Xenopus oocyte.

Fig. 1B - It is unclear how primordial follicle oocytes are distinguished from somatic cells, some of which are almost the same size. Labeling with a specific marker (like Vasa for example) would help determining this. Also, only a portion of the GV oocyte is shown, which makes the evaluation of the findings difficult.

Fig. S2 - The authors state: "We found that the Golgi ring had polarized Golgi stacks, and associated with pericentrin", however, the localization of the golgi apparatus around Pericentrin has already been reported by Lei and Spradling, 2016

Fig. 1C - It should be described and demonstrated how puncta were selected and measured for this analysis.

Fig. 1G - The insert shown hides the image of the oocyte, it is advisable to show the entire oocyte, so that readers can evaluate the results.

Fig. 1 H - the authors conclude that the mitochondria localization in the mouse oocyte is different than in humans and frog. To this reviewer it seems that mouse and human oocytes have similar mitochondria localization pattern, and that Xenopus is apparent the exception.

In entire figure 1, a use of Max Z projection seems less appropriate. A max projection will provide only the most intense signals in the oocyte. For a reliable evaluation of the distribution of the organelles shown, a Sum Z projection would recover all signals throughout the oocytes, allowing for better assessment of their distributions.

In addition, the authors heavily rely on dyes for their analyses. Vital dyes can be very useful, but are limited in tissue penetration for example, which could result in varying signal intensities between different parts of the tissue and between experiments. Validation by other methods, such as ultrastructure analysis and immune-fluorescent staining, or transgenically expressed or injected markers (for mouse and Xenopus, respectively) is required to support their conclusion.

Fig.2 - Based on the images shown, it is difficult to assess and compare the signal of the dye used, which shows substantial background. To my eyes, the data shown for mouse looks similar to that of the human oocyte. In addition, it is unclear how consistent and quantitative are the staining and imaging conditions that are used. The Xenopus image for example looks generally much brighter than the other species' oocytes. In addition, co-labeling for a landmark for the Balbiani body (like Pericentrin, golgi etc.,) would help to determine whether the shown signals originate from the Balbiani body versus the remaining cytoplasm.
This data as provided here does not support the conclusion that mouse Balbiani bodies do or do not contain amyloid structures.

In addition to the dye used, ultrastructure analysis is required. The authors also previously reported the use of a vital dye called ThT for identifying amyloid structures in live oocytes. It would be good to validate the data with such dye which also would help circumvent fixation artefacts.

"N"s are not provided for almost all experiments

Fig. 3 - The observation that mitochondria can localize to the position of the golgi is not convincing to conclude the lack of a Balbiani body or a proteinaceous structure. This experiment relies on three assumptions: 1. Proteins and organelles cannot be found closely adjacent to one another. However, in the Balbiani body of zebrafish and Xenopus, organelles are embedded within or surrounded by RNA-protein (RNP) granules. At the confocal resolution shown, one cannot conclude the lack of protein complexes that could still be present around the 'invading' mitochondria. 2. The golgi apparatus within a Balbiani body is not associated with such RNP granule. This was not ruled out. If this is the case, then dissociation of the golgi might also affect the structure of the Balbiani body and result with redistribution of mitochondria. 3. The mitochondria are not part of the Balbiani body. This was not proven. Balbiani body RNP can be associated with the 'invading' mitochondria, and in this case the results would not conclude a lack of Balbiani body. In fact, the RNP complexes that are found in piRNA granules in germ cells across vertebrate ad invertebrate species were described as nuage, electron-dense material (presumptive RNPs) that is associated with mitochondria. This is consistent with observations of intermingled RNP and mitochondria (and other organelles) in the Balbiani body in fish and frog.

In addition, no controls are provided to rule out non-specific effects in this experiment. In the measurement of the area occupied by mitochondria, the authors should specify whether this analysis was performed on single optical sections, or on Z projections.
Altogether, I am afraid that the rational and technical aspects of this experiment are not compelling to conclude a lack of a Balbiani body.

Fig. 4 - In the references mentioned, I could not find data showing the localization of RNGTT in the Balbiani body. RAP55 was shown to localize to the Balbiani body in the mouse, and it is possible that it represents a golgi marker and not necessarily a Balabiani body marker.
However, to rule out previous findings from Pepling et al., 2007, I would expect more thorough investigation, attempting to test RAP55 localization at different oocyte stages. Pepling et al, showed data from E14.5, PND1, PND3. To conclude that RAP55 is completely absent from the Balbiani body, would require examination at different stages in order to rule our transient localization.

It should be noted whether single sections or z projections are shown, and the entire volume of the golgi/Balbiani body regions, as well as this of the entire cytoplasm should be shown, or at least reported.

In general, it is possible that the RNA and protein of the Balbiani body would be different between different species. For example, Bucky ball, the only protein known to be essential for Balbiani body formation as found in zebrafish was reported to be rapidly evolving and does not have true homologs outside zebrafish and frogs (XVelo). I find it difficult to conclude a lack of Balbiani body in the mouse, based on the localization of these two specific proteins.

Fig. 5 - The images do not clearly represent the described text. In panel A bottom panel - 'Primary' seems to have no golgi ring. In panel C - the authors state examples of oocyte with and without golgi ring, but all panels seems to show one, except for the bottom row. This should be clarified or other representative images should be shown.

E-F - this analysis is difficult to interpret as bulk data. The data has to be more specifically analyzed and presented. For example, it would be ideally performed by first scoring oocytes for FOXO nuclear localization, blindly to the golgi organization. Then the oocytes selected for either FOXO localization group should be scored for the presence of a golgi ring. The percentage of oocytes with or without golgi ring then should be presented for each FOXO localization category.

This experiment is based on the claim that golgi ring disassembly by BFA disassembles the Balbiani body, which remains controversial. It may still be possible that BFA does not affect the Balbiani body per se, making the results from this experiment difficult to interpret. It is also possible that the disruption of the golgi cause non-specific effect. For example if Balbiani body dissociation normally effects FOXO localization via a protein that requires a functional golgi organelle for its processing. More controls are needed to avoid misinterpretations.

Panel B - The size and resolution of the images make it difficult to evaluate the formation of the golgi ring and FOXO localization. Images with increased size and resolution should be provided, as well as single channel images for each signal.

In Supp. Videos 3-6 the general brightness of signals seem to vary significantly. The authors should report on whether experimental conditions, including staining and image acquisition were kept uniform and consistent to allow for quantitative comparisons within and between experiments.

Minor Comments
Comment 1:

Several statements in the introduction are not accurate, as follows:
"The earliest stage of a recognizable oocyte in the ovary is the primordial oocyte" - this is not correct. Oocyte differentiation initiates with the induction of meiosis, which occurs while oocytes continue to develop within a germline cyst. In mammals, the oocyte forms the primordial follicle only at pachytene stages of meiosis I, well within its differentiation. Similar consistent dynamics was also reported in zebrafish.

"Primordial oocytes constitute the fixed ovarian reserve and are considered dormant as they do not grow nor divide" - this is not accurate. Primordial follicles develop to primary growing follicles and a subset pool of primordial follicles are arrested as the ovarian reserve.

"The Balbiani body is only present in early, dormant oocytes and dissociates upon oocyte activation." - This is not accurate. In Zebrafish the Blabiani body dissociates at late St. I of zebrafish oogenesis, well within primary follicle stages. In the mouse, the Balbiani body was observed in primary follicles in P7 postnatal ovaries at the latest. Moreover, in Zebrafish early Balbiani body formation was shown to begin at zygotene stages of meiosis I, while oocytes are still in the germline cyst (Elkouby et al., 2016). A similar early formation of the Balbiani body in early meiotic prophase (E14.5) germline cyst was shown in the mouse (Lei and Spradling, 2016). The oocyte is by no means 'dormant' at these stages. Altogether, stages that include the Balbiani body or its formation are not specific to primordial follicles.

Comment 2:

A better labeling of the species shown for panels in figures and cartoons would help orientation.

Significance

The subject of this investigation has potential for great interest by the communities of germ cell and developmental biologists, as well as cell biologists and reproduction biologists.
However, unfortunately, I am afraid that with the concerns raised above, the data has to substantially improve before it can be relevant for this audience.

The authors report a conclusion that goes against several published reports. This off course would be completely appropriate, but in such case I would expect the authors to perform a more thorough investigation and present more solid data, which currently are not compelling to rule out the said previous reports. In one case, the authors report a finding that was already published by others.

I have expertise in Balbiani body biology.

Author response to reviewers' comments

1. General Statements

In this manuscript, we developed methods **to study oocyte cell biology in living human primordial oocytes for the first time**, as far as we are aware, and compare these to two classic model systems for vertebrate development, *Xenopus* and mouse. We are delighted to hear that all three reviewers consider our work important and influential:

Reviewer 1: "…an **important elegant and detailed live-imaging study** of germplasm composition in Xenopus, mice and humans **which provides a true original knowledge**."

Reviewer 2: "The work is well-done, and **the findings are exciting and significant** to reproductive biologists investigating vertebrate oocyte biology, such as myself, and more broadly to cell biologists, developmental biologists, geneticists, and those investigating fertility and reproductive health."

Reviewer 3: "Understanding the formation, organization and various features of the Balbiani body is therefore **of great importance**."

Reviewer 1 and 2 are mostly complimentary in their comments, and suggested few additional experiments and some clarifications in the manuscript. We have already incorporated all their suggestions to the transferred manuscript. Reviewer 3 expresses substantially more skepticism, but provides much constructive criticism at both technical and philosophical levels. Reviewer 3 requests several experiments, most of which we have already performed and included in the current, transferred manuscript. Resubmission deadlines have prevented us from completing a subset of the more technically challenging experiments (detailed below) but we expect to complete them in the upcoming weeks.

In total, we believe that the large body of additional data serves to support our conclusions and strengthen our arguments. We thank all three reviewers for their feedback. We must note that reviewer 3 at a few specific points has made, in our opinion, statements about the Balbiani Body that appear in conflict with the decades-long literature. Reviewer 3 appears to assert that a Balbiani body could exist without mitochondrial conglomeration, as quoted below:

"The authors conclude the "mouse oocytes do not contain a Balbiani body"… It may very well be that the mouse Balbiani body is different than that of other species in its content and/or organization…"

"Fig. 3 - The observation that mitochondria can localize to the position of the golgi is not convincing to conclude the lack of a Balbiani body or a proteinaceous structure. This experiment relies on three assumptions: … 3. The mitochondria are not part of the Balbiani body…"

Reviewer 3's critique could be seen as an issue of semantics - what organelle merits being given the name "Balbiani Body"? In our manuscript we stick closely to the conventional definition of the term "Balbiani body" which dates back to the 1920s but has spawned several synonyms including "mitochondrial cloud", "mitochondrial mass", and "mitochondrial aggregate". Regardless of the name used, the essential characteristic of the Balbiani body has been a conglomeration of mitochondria in early oocytes (Banani et al., 2017; Bilinski et al., 2017; Boke et al., 2016; Bontems et al., 2009; Cox and Spradling, 2003; Guraya, 1979; Hertig, 1968; Hertig and Adams, 1967; Kloc et al., 2004; Marlow and Mullins, 2008; So et al., 2021; VAN DER STRICHT, 1890; Woodruff et al., 2018). To clarify any confusion, we now highlight the standard definition of the Balbiani body more obviously in the introduction.

Reviewer 3 additionally makes a more philosophical point that it's epistemologically difficult to prove the non-existence of anything, let alone an organelle. Though true, we emphasize that we not only fail to find a conventional Balbiani body in mouse early oocytes but also demonstrate how a structure previously claimed to be a Balbiani body is in fact a mis-identified Golgi cluster.

Therefore, in the transferred manuscript we continue to use the established definitions for the Balbiani body and more clearly reference similar uses of the term in the existing literature.

2. Description of the planned revisions

Insert here a point-by-point reply that explains what revisions, additional experimentations and analyses are planned to address the points raised by the referees.
We have already performed and addressed the majority of the revisions, additional experiments and analyses asked by the reviewers (please see section 3 below). However, we are still missing **three experiments and one re-analysis**, which we are hoping to perform and complete in the next few weeks:

1- We are in the process of performing the experiment below to strengthen our conclusions, encouraged by a comment (rather than point) of "Reviewer #1"

"…It is therefore an important elegant and detailed live-imaging study of germplasm composition in Xenopus, mice and humans which provides a true original knowledge. **It should be noted that oocytes were isolated from neonatal mice, young adult Xenopus and from women aged 18 to 37 years…"**

We agree with the reviewer that isolating primordial oocytes only from neonatal mice is not ideal for our analyses, considering that we compare them to young adult *Xenopus* and women. We initially preferred using neonatal mice because they are most commonly (in fact, almost exclusively these days) used in the literature to isolate primordial oocytes from mice (Castrillon et al., 2003; Eppig and Wigglesworth, 2000; Morohaku et al., 2016; Pepling et al., 2007; Shimamoto et al., 2019). We will repeat our live cell imaging experiments with primordial follicles isolated from young adult mouse (5-8 weeks) using the Golgi marker and incorporate these results to the text (experiments with mitochondria and lysosomal markers are already repeated in adult primordial oocytes and incorporated in the text, please see the corresponding paragraph in section 3).

2- We are in the process of performing this experiment including biological replicates, to address the point quoted below from Reviewer 3:

"Fig. 4 - .... RAP55 was shown to localize to the Balbiani body in the mouse, and it is possible that it represents a golgi marker and not necessarily a Balabiani body marker. However, to rule out previous findings from Pepling et al., 2007, I would expect more thorough investigation, attempting to test RAP55 localization at different oocyte stages. Pepling et al, showed data from E14.5, PND1, PND3. To conclude that RAP55 is completely absent from the Balbiani body, would require examination at different stages in order to rule our transient localization."

We are now in the process of providing this information from E14.5 and PND1 ovaries as asked by the reviewer. However, for now, we can speculate that RAP55 might be diffuse in E17.5 ovaries, but forms distinct puncta in P0, P1, and P2 ovaries - supported by DDX6 localization in the literature (Flemr et al., 2010; Kato et al., 2019), and our data that shows DDX6 and RAP55 colocalization (Figure 4D).

We also would like to note that Pepling et al., 2007 used two custom-made antibodies raised in the same species (rabbit) (Pepling et al., 2007; Wilhelm et al., 2005; Yang et al., 2006) to support their conclusions regarding colocalisation of Trailer hitch and RAP55, and have not shown any evidence regarding RAP55 localisation to the Golgi ring. Two primary antibodies raised in the same species are very likely to show spurious co-localisation, which would explain the discordance between our and previous findings.

3- We will incorporate the results of the co-labelling of Proteostat with mitochondria, a definitive landmark for the Balbiani body, as requested by the following comment of reviewer 3:

Reviewer 3:
Fig.2 - … In addition, co-labeling for a landmark for the Balbiani body (like Pericentrin, golgi etc.,) would help to determine whether the shown signals originate from the Balbiani body versus the remaining cytoplasm.

4- We would like to re-analyse the data in Figure 5E-F as asked by the reviewer, quoted below:

"E-F - this analysis is difficult to interpret as bulk data. The data has to be more specifically analyzed and presented. For example, it would be ideally performed by first scoring oocytes for FOXO nuclear localization, blindly to the golgi organization. Then the oocytes selected for either FOXO localization group should be scored for the presence of a golgi ring. The percentage of oocytes with or without golgi ring then should be presented for each FOXO localization category.

We will add an additional analysis performed as asked by the reviewer in a supplementary figure.

We foresee all of these experiments and the analysis will be completed before the end of September.

3. Description of the revisions that have already been incorporated in the transferred manuscript

Please insert a point-by-point reply describing the revisions that were already carried out and included in the transferred manuscript. If no revisions have been carried out yet, please leave this section empty.

Reviewer #1 (Evidence, reproducibility and clarity):

Laasya Dhandapani, et al have developed elegant methods for comparative characterization based on live imaging of primordial oocytes from Xenopus, mice and humans. Using specific markers for different intracellular organelles and pharmacological antagonists, they show that the three oocytes contain active mitochondria, Golgi apparatus and lysosomes. These organelles are included in a Balbiani body in Xenopus and human but not in mouse oocytes. In the latter they found in place of the Balbiani body a ring-shaped Gologi apparatus and the latter persisted when the oocytes were activated. The authors conclude that mouse oocytes do not contain a Balbiani body and that this structure is not associated with oocyte dormancy contradicting previous reports. This work is well done and interesting. However the fact that the oocyte of mice did not contain Balbiani body (Title) is not the main result of their work. This has already been shown in other species. It was shown from a long time that germ cells segregate from the soma early in development and that germ cell fate depends on the acquisition of a germ cell determinant commonly referred to as germplasma, an essential element of germ cell identity. In various animal species the subcellular composition of this germ plasm is different, and it has been shown that it contains various molecules such as RNA, proteins... Oocytes of certain species such as rats, hamsters, guinea pig, rabbits, nonhuman primates and humans have a Balbiani body similar in ultrastructure and behavior to those of Xenopus. But little information on their molecular composition and function is available. Mice oocytes on the contrary do not have any structure corresponding to the germ plasm, and no Balbiani body. The originality of this work is the comparative analysis of the subcellular composition of the oocytes of these 3 species. The authors show that all three oocytes contain mitochondria, Golgi apparatus, and that all organelles are functional. It was previously postulated that healthy mitochondria selectively associated with the Balbiani body to allow removal of defective mitochondrial genomes from ultimate germ cells.

We thank the reviewer for the positive comments and the encouragement. In light of their comments, we decided to change the title of our manuscript to:

"Comparative analysis of vertebrates reveals that mouse primordial oocytes do not contain a Balbiani body".

Reviewer #1 (Significance (Required)):

The authors show that the ring-shaped Golgi apparatus in mice is not linked to a dormant state of the oocyte. It is known that the Balbiani body or related structure is characteristic of the formation of primordial germ cells which cannot develop when this structure is altered. However the link with a dormant state of the oocyte has not been the main opinion in the literature (see for example the recent reviews Chun So et al, Trends Cell Biology, 2021,31, 254-267 or Malgorzata Kloc et al,

Current topics in Developmental Biology, 2004, 59, 1-36)..). However the authors do not study the apparition of these structures during development and the possible consequences of blocking different organelles on the subsequent development or function of the oocyte. It was previously hypothesized that the appearance and persistence of a Balbiani body was closely related to the type of the ovary and length of the oogenesis, and appears when oogenesis is rather slow but not in rapid oogenesis because the formation and transport of germplasm component occur rapidly (above References).

It is therefore an important elegant and detailed live-imaging study of germplasm composition in Xenopus, mice and humans which provides a true original knowledge. It should be noted that oocytes were isolated from neonatal mice, young adult Xenopus and from women aged 18 to 37 years. Further imaging and functional studies are needed to understand the function of the Balbiani body or the ring-shaped Golgi apparatus in germ cell growth, differentiation polarity and fertilization.

We agree with the reviewer that isolating primordial oocytes only from neonatal mice is not ideal for our analyses, considering that we compare them to young adult *Xenopus* and women. We initially preferred using neonatal mice because they are most commonly (in fact, almost exclusively these days) used in the literature to isolate primordial oocytes from mice (Castrillon et al., 2003; Eppig and Wigglesworth, 2000; Morohaku et al., 2016; Pepling et al., 2007; Shimamoto et al., 2019). However, we repeated our live-cell imaging experiments with primordial follicles isolated from young adult mouse (5-8 weeks) using markers for lysosomes and mitochondria. Primordial follicles isolated from adult mice also displayed active lysosomes and mitochondria, repeating similar patterns we observed in primordial follicles isolated from neonatal mice. We incorporated these results as a supplementary figure (Figure S4A), and inserted the following text:

"Primordial oocytes are almost exclusively obtained from neonatal mice in the recent literature (Shimamoto et al., 2019; Pepling et al., 2007; Morohaku et al., 2016; Castrillon et al., 2003; Eppig, et al., 2000). Since *Xenopus* and human oocytes in this study were isolated from young adults, we repeated our experiments using primordial oocytes obtained from young adult mice. We found that similar to newly formed primordial oocytes, young adult primordial oocytes also contained active mitochondria and lysosomes. Thus, primordial oocytes isolated from neonatal and young adult mice are similar with regards to the activity of their organelles (Figure S4A)."

We thank the reviewer for bringing this up, which certainly improved our manuscript.

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

Asymmetric localization of cellular components is a shared feature of early oocytes. The Balbiani body is a nonmembrane bound compartment that has been observed in species ranging from insects to vertebrates, including mammals. This structure has been postulated to play a role in regulating oocyte dormancy, RNA localization and regulation, and sequestration of determinants, such as the germ plasm and patterning molecules in some animals, and allocation or sorting of cellular organelles, namely mitochondria. In recent years, the Balbiani body has been shown to assemble by a mechanism that involves intrinsically disordered proteins and amyloid formation. In this work the primary oocytes of three species, Xenopus, mouse, and human are characterized using live imaging, vital dyes, and inhibitors to probe the distribution and activity of cellular organelles. The live imaging approach that sets this work apart from previous studies examining cellular asymmetries in mouse, Xenopus, and human oocytes. Interestingly, this study revealed that lysosomes are active in vertebrate oocytes. In contrast, lysosomes are not thought to be active in the invertebrates examined thus far. The manuscript is well written and has several new and exciting findings, including a more detailed comparison of asymmetries and organelles within vertebrate oocytes that challenges the existing models for mammalian Balbiani body composition that were based largely on the mouse oocyte. Overall, the experiments appear to be well controlled, the data are of high quality, and the data provided largely support the conclusions, but could be strengthened by attending to some easily addressable matters, center around missing or unclear numbers for some of the experiments and that some of the analysis appears to have only been quantified in the mouse. Given that this is a comparative study, and that a key conclusion is that the mouse is different, the supporting quantitative data should be provided.

We thank the reviewer for these positive comments! We provide the quantitative data in this revised version, please see below.

Major:

One potential concern is that the human oocytes used were obtained from women who had undergone ovarian surgery. If the women had surgery because of cysts, cancer, or similar reproductive health concerns, then it is possible that the oocytes may not represent are healthy normal oocytes. If this is a possibility, then this should be stated. It is probably a good idea to clarify even if it is not a concern - if these were all or in part from healthy women free of disease affecting the reproductive system.

We thank the reviewer for highlighting this, indeed we only used oocytes from women free of disease affecting the reproductive system. We now include the full study conditions, and the following statement in methods:

"Women fulfilling the inclusion criteria undergoing ovarian surgery were asked to participate in the study. Informed consent was obtained from all of them.
Inclusion criteria: Age between 18 to 35, fertile (assessed by un-induced menstrual cycles or presence of antral follicles identified by ultrasound examination), presence of at least one ovary, signed informed consent.
Exclusion criteria: women with menopause, endometriosis, or who underwent bilateral oophorectomy.

All oocytes incorporated in this study were from women free of disease affecting the reproductive system."

In Figure 1 panel C quantification of lysotracker is shown for mouse oocytes. Was similar analysis performed on the lysotracker labeled Xenopus and human oocytes? Including this analysis would provide a quantitative basis for comparison of these different oocytes.

We thank the reviewer for this suggestion; we now include Lysotracker intensity measurements for all three species, comparing puncta in oocytes and in the (somatic) follicle cells surrounding them (Figure 1B). We modified the main text accordingly.

Based on the TMRE staining shown in Figure 1H, the mouse and human oocytes appear similar. Both appear to have domains devoid of mitochondria? For the Xenopus and human oocyte analyses, how many oocytes were examined? From how many females? It would be helpful to include the specific quantification and criteria that were used to conclude that mitochondria distribution is different in human and mouse oocytes- Based on the examples shown, human looks quite different from Xenopus and more similar to the mouse oocyte. Is this a function of stage? The schematics in panel I are helpful, but please add labels above to indicate the species.

We thank the reviewer for bringing this up, which gives us a chance to clarify our discovery. First of all, at least three individuals were examined for each species; eight human, twenty-three mouse and nine *Xenopus* oocytes were counted for mitochondrial staining. We now include this information in the figure legend.
The distribution of mitochondria in mouse and human oocytes looks, indeed, similar to each other and different from *Xenopus* at a first glance. However, a careful examination unraveled superficial similarities and highlighted features that are not obvious in the first place, mostly because of the major size differences of oocytes in these three species. Our examination concentrated on two basic points:

1- **Mitochondrial Conglomeration:** In human oocytes, the majority of the mitochondria is conglomerated adjacent to the nucleus, as in *Xenopus* oocytes. However, in mouse oocytes, mitochondria are distributed throughout the available space in the cytoplasm. In order to provide a quantification for this statement, we now calculated the center of mitochondrial mass in *Xenopus*, mouse and human oocytes: in mouse, the center of mitochondrial mass is located within the nucleus as mitochondria are distributed throughout the cytoplasm. In humans and *Xenopus*, the center of mitochondrial mass

remains within the Balbiani body or at the nucleus-Balbiani body border, as the majority of the mitochondria in these cells are conglomerated inside the Balbiani body. We added this analysis as an additional supplementary figure (Figure S3B, C).

2- **Not every cellular space not occupied by mitochondria is the same:** In mouse oocytes, we (and others) show that the distinct space in the cytoplasm that is not occupied by mitochondria is filled by the Golgi ring. In human oocytes, the "gap" in mitochondrial conglomeration (Figure S3G) has been observed numerous times in the literature, and corresponds to cytoplasmic membranes of unknown nature (Hertig, 1968; Hertig and Adams, 1967). We do see this gap in mitochondrial staining all the time in *Xenopus* oocytes too (Figure S3F, G) but the large size of these cells makes these gaps in mitochondrial staining look trivial. We now provide a side-by-side comparison of different sizes of oocytes and of this observed "gap" in mitochondrial staining for all three species (Figure S3F, G). Our previous electron microscopy images in Xenopus (Boke et al., 2016) and previous literature in human oocytes (Hertig, 1968) indicate that these membranes **do not** correspond to Golgi membranes which have very characteristic features. On the other hand, electron microscopy studies in mouse oocytes show that this gap is occupied with Golgi membranes (Lei and Spradling, 2016; Pepling et al., 2007).

Thus, we conclude that although mouse and human oocytes look similar at first sight, these similarities are likely superficial. A same scale image of oocytes from the three vertebrates (Figure S3F, G) highlights the importance of comparing these cells quantitatively.

It is not clear how many oocytes and individuals were examined for the proteostat experiments.

We apologise for this oversight. Three individuals were examined for each species; at least three oocytes were imaged for each human, ten oocytes for each mouse and five oocytes for each *Xenopus*. These numbers are now incorporated into the figure legend of the corresponding figure.

Minor:

A general comment for the graphs in figures where oocytes of more than one species are shown, please include labels indicating which species is shown. If a graph or schematic contains data from more than one species, please add a key.

We added this information for all the figures that have oocytes from different species.

The title for Figure 2 concludes that mitochondria of mouse primordial oocytes are not maintained within a Balbiani body, but mitochondria are not analyzed in the figure. Please consider revising to reflect the data shown or include data on mitochondria.

We have changed the figure title to "Mouse primordial oocytes do not contain a large proteinaceous matrix".

Scale bars are missing in Figure 3A, for the enlarged panels and insets in Figure 4, Supp. Fig 1A top row, and Supp. 4A

We apologise for these oversights; scale bars are now added to all of the indicated figures.

In Supp. Fig 1C and Supp. Fig. 2 - assume the samples shown are mouse but this is not indicated.

We added the relevant species information to all figures.

Supp. Fig. 2C - please show each sample point sas in the other figures.

We have changed the format of the figure such that we show the data points.

Reviewer #2 (Significance (Required)):

Previous analysis of mouse primary oocytes identified a Golgi ring as the mouse Balbiani body. Based on the lack of mitochondria accumulation in this structure, it was suggested that the

mammalian Balbiani body was unique from those of nonmammalian vertebrates. A striking finding from this work is that the mouse indeed appears to be unique but that somewhat unexpectedly, the primary oocytes of Xenopus and human appear to share similar cellular structures. Specifically, the distribution of organelles is different in mouse oocytes, and they appear to lack Balbiani bodies based on several criteria examined in this study: 1) no accumulation of mitochondria, 2) no evidence for RNA localization within a nonmembrane bound compartment, and 3) no evidence for an amyloid-like matrix. Moreover, staining and inhibitor treatments indicate that the Golgi ring has the hallmarks of a conventional Golgi structure and has no correlation to or instructive role in preventing or promoting oocyte activation, but does disassemble in response to oocyte activation. This work challenges the notion that mice even have a Balbiani body and provides evidence against the prevailing model that the Golgi ring regulates oocyte dormancy. The findings point to the importance of choosing a system to model a process based on a comparative cell biology approach rather than solely on evolutionary distance. The work is well- done, and the findings are exciting and significant to reproductive biologists investigating vertebrate oocyte biology, such as myself, and more broadly to cell biologists, developmental biologists, geneticists, and those investigating fertility and reproductive health

We thank the reviewer for these positive and encouraging comments.

Reviewer #3 (Evidence, reproducibility and clarity (Required)):
In this manuscript the authors aim to compare the content and organization of the Balbiani body between Xenopus, mouse and human oocytes. The Balbiani body is a conserved feature of oocytes across the animal kingdom. In frog and fish the Balbiani body is essential for oocyte polarity, proper fertilization and inheritance of maternal regulators that induces the dorsal-ventral axis of the future embryo, as well as its germline. In mouse, the Balbiani body has been associated with culling of successful oocytes during early formation of the primordial and primary follicle. Understanding the formation, organization and various features of the Balbiani body is therefore of great importance. While the Balbiani body has been extensively functionally studied in frog and fish, this aim is very important in mammals where functional tools for Balbiani body investigation are not yet available.

Based on vital dye labeling the authors examine the organization of the Balbiani body and conclude that mouse oocytes do not contain a Balbiani body. Unfortunately, I have substantial concerns regarding the experiments shown and I could not find the data compelling to support such a conclusion. Generally, the authors heavily rely on the use of vital dyes and do not employ complementing methodologies or approaches to support these findings. The rational of some of the experiments is not clear and rely on weak assumptions and some controls are missing. In addition, the number of oocytes/ovaries examined is rarely provided, and the presentation of the data is not always clear. I am detailing my comments below.

There are several studies performed on fixed ovary tissues in the literature (Castrillon et al., 2003; Kato et al., 2019; Lei and Spradling, 2016; Shimamoto et al., 2019). Although these studies have been pivotal in nature, and pushed the field forward in many ways, they have been limited by common problems associated with fixation: loss of information on organelle dynamics and activity; introduction of fixation and permeabilization artefacts such as the distortion of cell shape, hindering of epitopes that would be recognized by the antibody and the loss of membrane proteins. Moreover, a reliance on antibodies makes it very challenging to detect oocyte/germ cell- specific splice variants to mark organelles, and some species-specific proteins.

In fact, it is still impossible to find a non-sectioned or not fixed image or description of mammalian primordial oocytes in the literature. As the other reviewers also pointed out, the pillar of our study is live-cell imaging: only by live-cell imaging we can collect information regarding the activity of organelles and their dynamic distribution in the cytoplasm. However, we would like to point out that we have extensive immunofluorescence images with whole-ovary imaging (our preferred fixation method to avoid shape-distortions observed during fixation in large cells like oocytes) and in some cases, tissue sections (Figure 4, 5, S2, S4, S7).

The authors conclude the "mouse oocytes do not contain a Balbiani body". As a constructive advice, I would like to comment that it is extremely challenging conceptually to prove that something does not exist. It may very well be that the mouse Balbiani body is different than that of other species

in its content and/or organization, and I believe that the authors should consider this possibility.

We thank the reviewer for their comments. The name Balbiani body, or “mitochondrial cloud” as it was referred to as in older literature, is used to describe a membraneless super-organelle that clusters mitochondria (Bilinski et al., 2017; Guraya, 1979; Hertig, 1968; Jamieson-Lucy and Mullins, 2019; Kloc et al., 2004). Because mouse oocytes lack mitochondrial conglomeration and do not contain a proteinaceous matrix indicative of a membraneless super-organelle -two defining characteristics of the Balbiani body- we conclude that mouse oocytes lack a Balbiani body. Furthermore, ours is not merely a negative finding; we positively identify the structures previously described as Balbiani bodies as in fact mis-identified Golgi rings: the structures formerly known as murine Balbiani bodies do not fulfill the criteria of a Balbiani body, as they associate neither with a proteinaceous matrix (Figure 2, S5), nor mitochondria (Figure 3, S6), nor with known RNA-binding proteins (Figure 4). Therefore, our contribution is not only to “prove that something does not exist” but it is also to prove that existing structures have been misidentified. Of course, there are epistemological limitations to any experiment: we cannot exclude the possibility that some currently unknown functions performed by the Balbiani body in humans and *Xenopus* may be performed by a structurally distinct organelle in mouse oocytes that does not fulfil any of the above criteria. However, this structure, then, would not be a Balbiani Body.

We would like to note that it is not so unusual for an organelle to be missing from a particular cell type. There are many examples, such as cells that lack centrosomes (Bornens, 2012; Schuh and Ellenberg, 2007) or mitochondria (Muller, 1988). Such cell types happen to contain types of centrosomal or mitochondrial material to organize their acentrosomal spindle assembly or electron transport function, respectively, but these are not assembled and so the cells lack the namesake organelles. In a similar way, we have shown that mouse oocytes do not contain a Balbiani body.

Major Comments

Fig. 1A - LysoTracker signal in the Xenopus oocyte looks more like background than a true signal. Also, the insert hides much of the shown oocyte, it is advisable to show the entire oocyte. Based on the images shown it is difficult to conclude that "vertebrate primordial oocytes have acidic, active lysosomes distributed in their cytoplasm" for the Xenopus oocyte.

Xenopus stage I oocytes are huge (250 microns), whereas lysosomes are very small (0,2-1,2 microns in diameter). Thus, we understand the reviewer’s concern that the lysosomal puncta might come across as background staining without additional magnification.
We now address this concern by

1- including full-size, high-magnification images of *Xenopus* oocytes (Figure S1B).

2- performing an additional experiment with Bafilomycin A1, a lysosome acidification inhibitor, to prove the specificity of the Lysotracker dye in *Xenopus* oocytes too. In the presence of Bafilomycin A1, lysosomes do not uptake Lysotracker and thus, do not show any specific Lysotracker staining (Figure S1C).

We thank the reviewer for their suggestion to make this conclusion stronger.

We also provide additional quantifications of lysosomal puncta inside the oocyte and in somatic cells surrounding the oocyte for all three species. The Lysotracker intensity in each follicle was similar between somatic cells and the oocyte of that follicle (Figure 1B), providing additional vigor for our conclusion that “vertebrate primordial oocytes have acidic, active lysosomes distributed in their cytoplasm”

Fig. 1B - It is unclear how primordial follicle oocytes are distinguished from somatic cells, some of which are almost the same size. Labelling with a specific marker (like Vasa for example) would help determining this. Also, only a portion of the GV oocyte is shown, which makes the evaluation of the findings difficult.

It is well-established to differentiate and classify oocytes according to their size and morphology in both mammalian and frog reproduction fields (Clarke, 2012; Dumont, 1972; Gougeon, 1986; Morohaku et al., 2016; Pedersen and Peters, 1968; Wang et al., 2017; Westergaard et al., 2007).

Thus, we now included the size information for oocytes in the Materials and Methods section of the manuscript in the paragraph ”Oocyte classification” and labelled primordial and growing oocytes in the figure. Moreover, since a DDX4 staining (Vasa homolog in mammals) would require fixation and would therefore not be compatible with LysoTracker staining, we show here images of oocytes isolated from transgenic Stella-GFP mice (a germ cell marker) (Payer et al., 2006) labelled with LysoTracker, replicating our previous results and conclusions.

***NOTE**: We have removed unpublished data that had been provided for the referees in confidence.*

Fig. S2 - The authors state: "We found that the Golgi ring had polarized Golgi stacks, and associated with pericentrin", however, the localization of the golgi apparatus around Pericentrin has already been reported by Lei and Spradling, 2016

We apologise for this oversight; we have changed our wording to:
“We found that the Golgi ring had polarized Golgi stacks, and confirmed previous reports that it is associated with pericentrin".

Fig. 1C - It should be described and demonstrated how puncta were selected and measured for this analysis.

We added this information as an additional supplementary file (please see Figure S1D) and expanded the materials and methods with detailed description.

Fig. 1G - The insert shown hides the image of the oocyte, it is advisable to show the entire oocyte, so that readers can evaluate the results.

We now include all images of *Xenopus* oocytes and their magnifications as supplementary figures in full size (Figure S1B). We also provide a to-the-scale comparison of *Xenopus,* human and mouse oocytes in supplementary figure 3F to highlight the scale differences of these cells. Unfortunately, for space concerns, we had to keep the inserts in the main figures.

Fig. 1 H - the authors conclude that the mitochondria localization in the mouse oocyte is different than in humans and frog. To this reviewer it seems that mouse and human oocytes have similar mitochondria localization pattern, and that Xenopus is apparent the exception.

We thank the reviewer for bringing this up, which gives us a chance to clarify our discovery. First of all, at least three individuals were examined for each species; eight human, twenty-three mouse and nine *Xenopus* oocytes were counted for mitochondrial staining. We now include this information in the figure legend.
The distribution of mitochondria in mouse and human oocytes looks, indeed, similar to each other and different than *Xenopus* at a first glance. However, a careful examination unraveled superficial similarities and highlighted features that are not obvious in the first place, mostly because of the major size differences of oocytes in these three species. Our examination concentrated on two basic points:

1- **Mitochondrial Conglomeration:** In human oocytes, the majority of the mitochondria is conglomerated adjacent to the nucleus, as in *Xenopus* oocytes. However, in mouse oocytes, mitochondria are distributed throughout the available space in the cytoplasm. In order to provide a quantification for this statement, we now calculated the center of mitochondrial mass in *Xenopus*, mouse and human oocytes: in mouse, the center of mitochondrial mass is located within the nucleus as mitochondria are distributed throughout the cytoplasm. In humans and *Xenopus*, the center of mitochondrial mass remains within the Balbiani body or at the nucleus-Balbiani body border, as the majority of the mitochondria in these cells are conglomerated inside the Balbiani body. We added this analysis as an additional supplementary figure (Figure S3B, C).
2- **Not every cellular space not occupied by mitochondria is the same:** In mouse oocytes, we (and others) show that the distinct space in the cytoplasm that is not occupied by

mitochondria is filled by the Golgi ring. In human oocytes, the "gap" in mitochondrial conglomeration (Figure S3G) has been observed numerous times in the literature, and corresponds to cytoplasmic membranes of unknown nature (Hertig, 1968; Hertig and Adams, 1967). We do see this gap in mitochondrial staining all the time in *Xenopus* oocytes too (Figure S3F, G) but the large size of these cells make these gaps in mitochondrial staining look trivial. We now provide a side-by-side comparison of different sizes of oocytes and of this observed "gap" in mitochondrial staining for all three species (Figure S3F, G). Our previous electron microscopy images in *Xenopus* (Boke et al., 2016) and previous literature in human oocytes (Hertig, 1968) indicate that these membranes **do not** correspond to Golgi membranes which have very characteristic features. On the other hand, electron microscopy studies in mouse oocytes show that this gap is occupied with Golgi membranes (Lei and Spradling, 2016; Pepling et al., 2007).

Thus, we conclude that although mouse and human oocytes look similar at first sight, these similarities are likely superficial. A same scale image of oocytes from the three vertebrates (Figure S3F, G) highlights the importance of comparing these cells quantitatively.

In entire figure 1, a use of Max Z projection seems less appropriate. A max projection will provide only the most intense signals in the oocyte. For a reliable evaluation of the distribution of the organelles shown, a Sum Z projection would recover all signals throughout the oocytes, allowing for better assessment of their distributions.

We provide sum-Z projections below. Upon preparing sum slices of the images (follicles are roughly ~25, 50 and 250 microns, in mouse, humans, and *Xenopus*, respectively) the signal within the primordial follicle was over-saturated in all 3 species. The resulting image was not clear and the organelle distribution could not be discerned especially in the case of Lysotracker and NBD C_6 Ceramide probes, which are membrane-soluble and tend to accumulate weakly in all membranes. Hence, we prefer using max-Z projections in the manuscript.

***NOTE**: We have removed unpublished data that had been provided for the referees in confidence*.

In addition, the authors heavily rely on dyes for their analyses. Vital dyes can be very useful, but are limited in tissue penetration for example, which could result in varying signal intensities between different parts of the tissue and between experiments. Validation by other methods, such as ultrastructure analysis and immune-fluorescent staining, or transgenically expressed or injected markers (for mouse and Xenopus, respectively) is required to support their conclusion.

We thank the reviewer for their concern. We provide below diffusion calculations for biological systems, considering the size of oocytes, and their attached somatic cells (pasted below):

NOTE: We have removed unpublished data that had been provided for the referees in confidence.

Here were used the equation for one-dimensional mean square displacement of a live-cell imaging probe (Berg, 2018) assuming lipid-like property and spherical symmetry of the oocyte to obtain the time for diffusion of the probe (t) as:

$$t = \frac{r^2}{2D}$$

Where r is the radius of the somatic cell and oocyte that comprise the follicle, D is the diffusion constant which was assumed to be 5μm^2/sec for a lipid diffusing through the membrane and 50 μm^2/sec for a lipid diffusing through cytoplasm. The total diffusion time is calculated as:

$$t_{total} = t_{PM1} + t_{somatic\ cell\ cytoplasm} + t_{PM2} + t_{OPM} + t_{oocyte\ cytoplasm}$$

Where PM1 represents the somatic cell plasma membrane facing the medium in which the dye is dissolved, PM2 is the plasma membrane of the somatic cell in contact with the oocyte and OPM is oocyte plasma membrane. An example for the diffusion through a mouse follicle is shown:

NOTE: We have removed unpublished data that had been provided for the referees in confidence.

As the reviewer can see, even in the case of large *Xenopus* oocytes, we are not limited by diffusion (Table 1). Moreover, all our labelling experiments provided similar or higher intensity of fluorescence inside oocytes compared to that of the surrounding somatic cells, which would go against limited penetration.

Finally, as suggested by the reviewer, we now provide in a supplementary figure (Figure S4B-D) immunofluorescence images of primordial oocytes performed in fixed tissue sections of the three vertebrates supporting our findings using live-imaging regarding the distribution of organelles.

Fig.2 - Based on the images shown, it is difficult to assess and compare the signal of the dye used, which shows substantial background. To my eyes, the data shown for mouse looks similar to that of the human oocyte. In addition, it is unclear how consistent and quantitative are the staining and imaging conditions that are used. The Xenopus image for example looks generally much brighter than the other species' oocytes. In addition, co-labeling for a landmark for the Balbiani body (like Pericentrin, golgi etc.,) would help to determine whether the shown signals originate from the Balbiani body versus the remaining cytoplasm. This data as provided here does not support the conclusion that mouse Balbiani bodies do or do not contain amyloid structures. In addition to the dye used, ultrastructure analysis is required. The authors also previously reported the use of a vital dye called ThT for identifying amyloid structures in live oocytes. It would be good to validate the data with such dye which also would help circumvent fixation artefacts.

"N"s are not provided for almost all experiments.

We understand the reviewer's concern, and now provide a thresholding analysis on the images that clearly shows that human and *Xenopus* oocytes have localized Proteostat staining in their cytoplasm (top 10% fluorescent intensity thresholding masks applied), whereas mouse oocytes do not (Figure S5A). In fact, we only observe nucleoli distinctly in mouse oocytes, which serve as a positive control (as nucleoli are phase separated compartments and shown to contain amyloid-like material in some species, or under certain circumstances (Alberti and Carra, 2019; Frottin et al., 2019; Hayes and Weeks, 2016). Thresholding is an objective criterion (as opposed to what is visible to an eye), and clearly shows that *Xenopus* and human oocytes have cytoplasmic Proteostat-positive assemblies in the shape of the Balbiani body, whereas mouse oocytes only have cytoplasmic granules distributed in their cytoplasm. We also incorporate zoomed out images from mouse and human ovaries that make the proteostat staining of Balbiani bodies (or lack- there-of) more apparent (Figure S5B).
However, as mentioned in paragraph 2, we will incorporate the results of the co-labelling of Proteostat with mitochondria, a definitive landmark for the Balbiani body as requested by the reviewer.

Ultrastructure analyses were already performed in the literature for all three vertebrate oocytes (please see below). The available data is of high quality and support our conclusions:

- In humans, apart from a mitochondrial conglomeration (Baca and Zamboni, 1967; Hertig, 1968; Hertig and Adams, 1967), "fibrils" were noted in the Balbiani body:
  "The prominent center, measuring up to 4.5 μm in diameter (…) is composed of electron opaque deposits embedded within a matrix of fine fibrils. These dense granules may become periodically aligned to form radiating fibrils which apparently merge with peripheral coarse fibers measuring up to 0.13 μm in diameter." (Hertig, 1968)
- In *Xenopus*, we and others showed mitochondrial conglomeration in oocyte cytoplasm without a surrounding membrane (Boke et al., 2016; Kloc et al., 2004).
- In mouse, Pepling et al., and others observed a diffuse mitochondrial distribution in

the cytoplasm and a Golgi ring (Pepling et al., 2007; Wischnitzer, 1967; Wischnitzer, 1970), different than frog and human oocytes.

We apologize for not including N numbers; they are now included in the figure legends. Unfortunately, due to the effect of Covid19 pandemic on elective surgeries, it is currently very difficult to receive human ovary samples and thus, we cannot provide live imaging of human oocytes with ThT. However, we include below live ThT staining of mouse primordial oocytes and frog oocytes with ThT. *Xenopus* oocytes have a ThT positive conglomeration in their cytoplasm (the Balbiani body), whereas mouse oocytes only have diffuse ThT staining.

Fig. 3 - The observation that mitochondria can localize to the position of the golgi is not convincing

to conclude the lack of a Balbiani body or a proteinaceous structure. This experiment relies on three assumptions: 1. Proteins and organelles cannot be found closely adjacent to one another. However, in the Balbiani body of zebrafish and Xenopus, organelles are embedded within or surrounded by RNA-protein (RNP) granules. At the confocal resolution shown, one cannot conclude the lack of protein complexes that could still be present around the 'invading' mitochondria. 2. The golgi apparatus within a Balbiani body is not associated with such RNP granule. This was not ruled out. If this is the case, then dissociation of the golgi might also affect the structure of the Balbiani body and result with redistribution of mitochondria. 3. The mitochondria are not part of the Balbiani body. This was not proven. Balbiani body RNP can be associated with the 'invading' mitochondria, and in this case the results would not conclude a lack of Balbiani body. In fact, the RNP complexes that are found in piRNA granules in germ cells across vertebrate ad invertebrate species were described as nuage, electron-dense material (presumptive RNPs) that is associated with mitochondria. This is consistent with observations of intermingled RNP and mitochondria (and other organelles) in the Balbiani body in fish and frog.

In addition, no controls are provided to rule out non-specific effects in this experiment.

In the measurement of the area occupied by mitochondria, the authors should specify whether this analysis was performed on single optical sections, or on Z projections. Altogether, I am afraid that the rational and technical aspects of this experiment are not compelling to conclude a lack of a Balbiani body.

We thank the reviewer for giving us an opportunity to clarify the rationale behind this experiment. The only assumption we rely on is that the compounds we use to dissociate the Golgi apparatus do not act on the hypothetical protein matrix. We believe that this assumption is correct for 2 reasons: First, we have used two compounds with two completely different modes of action to dissociate the Golgi apparatus: BFA, a compound long hailed for its specificity to act on the Golgi apparatus (Chardin and McCormick, 1999) and Nocodazole, a microtubule drug that is widely used in the literature to dissociate the Golgi (Cole et al., 1996; Turner and Tartakoff, 1989). Second, BFA does not have any effect on the Balbiani body in *Xenopus* oocytes (as shown in Figure 1F). An elegant study already showed that Nocodazole (for that matter, complete disruption of microtubules) does not have any effect on the structure of the *Xenopus* Balbiani body (Chang et al., 2004).

To specifically answer the reviewer's points above:

1. Proteins and organelles cannot be found closely adjacent to one another. However, in the Balbiani body of zebrafish and Xenopus, organelles are embedded within or surrounded by RNA-protein (RNP) granules. At the confocal resolution shown, one cannot conclude the lack of protein complexes that could still be present around the 'invading' mitochondria.

We did not assume organelles cannot be adjacent to each other. On the contrary, the mitochondrial exclusion zone (MEZ) calculations in this very figure rely on organelles moving and mixing (in case of mitochondria, through fusion and fission) with each other in the available cytoplasm to fill the place generated by the dissociated Golgi apparatus.

2. The golgi apparatus within a Balbiani body is not associated with such RNP granule. This was not ruled out. If this is the case, then dissociation of the golgi might also affect the structure of

the Balbiani body and result with redistribution of mitochondria

It is certainly a possibility, that the Golgi apparatus is associated with an RNP granule; especially considering proteins like Trailer hitch, the RAP55 orthologue in *Drosophila*, resides at ER-exit sites (Wilhelm et al., 2005), where the Golgi apparatus tends to be. However, phase-separated compartments are de-mixed from the surrounding cytoplasm, and in case of the Balbiani body, they have a solid or amyloid-like nature (Banani et al., 2017; So et al., 2021; Woodruff et al., 2018). Thus, a large proteinaceous matrix should be present where the Balbiani body is. Therefore, two pieces of evidence go against the likelihood of the statement above:

1- The Proteostat did not detect any phase-separated compartment in mouse oocytes, whereas it clearly marked the Balbiani body of human and X*enopus* oocytes (Figure 2, S5). Thus, it is highly unlikely that there is a proteinaceous matrix associated with the Golgi ring in mouse oocytes.

2- A large proteinaceous phase-separated compartment is unlikely to be dissociated by the dissociation of the Golgi apparatus it has been associated with. This certainly has not been observed before in the literature. Supporting evidence for this statement is that we already showed in our manuscript that the *Xenopus* Balbiani body is not affected by dissociation of the Golgi apparatus (Figure 1F).

3. The mitochondria are not part of the Balbiani body. This was not proven. Balbiani body RNP can be associated with the 'invading' mitochondria, and in this case the results would not conclude a lack of Balbiani body.

We have shown in Figure 3 and Figure S6 that mitochondria are not associated with the Golgi ring, and thus, are not part of a Balbiani-like compartment in oocytes. Considering the definition of a Balbiani body, or mitochondrial cloud as it has been referred to earlier, always include conglomeration of mitochondria, not having a mitochondrial conglomeration (irrespective of its association with the Golgi ring), is enough to conclude that mouse oocytes do not have a Balbiani body.

In the measurement of the area occupied by mitochondria, the authors should specify whether this analysis was performed on single optical sections, or on Z projections.

The MEZ calculations were performed on the equatorial sections of the oocytes.

Fig. 4 - In the references mentioned, I could not find data showing the localization of RNGTT in the Balbiani body.

We apologise for the oversight and now include the reference to the preprint regarding RNGTT localisation to the mouse Balbiani body (Lei et al., 2020).

RAP55 was shown to localize to the Balbiani body in the mouse, and it is possible that it represents a golgi marker and not necessarily a Balabiani body marker. However, to rule out previous findings from Pepling et al., 2007, I would expect more thorough investigation, attempting to test RAP55 localization at different oocyte stages. Pepling et al, showed data from E14.5, PND1, PND3. To conclude that RAP55 is completely absent from the Balbiani body, would require examination at different stages in order to rule our transient localization.

We are now in the process of providing this information from E14.5, and PND1 ovaries as asked by the reviewer. However, for now, we can speculate that RAP55 might be diffuse in E17.5 ovaries, but forms distinct puncta in P0, P1, and P2 ovaries - supported by DDX6 localization in the literature (Flemr et al., 2010; Kato et al., 2019), and our data that shows DDX6 and RAP55 colocalization (Figure 4D).

We also would like to note that Pepling et al., 2007 used two custom-made antibodies raised in the same species (rabbit) (Pepling et al., 2007; Wilhelm et al., 2005; Yang et al., 2006) to support their

conclusions regarding colocalization of Trailer hitch and RAP55, and have not shown any evidence regarding RAP55 localization to the Golgi ring. Two primary antibodies raised in the same species are very likely to show spurious colocalization, which would explain the discordance between our and previous findings.

It should be noted whether single sections or z projections are shown, and the entire volume of the golgi/Balbiani body regions, as well as this of the entire cytoplasm should be shown, or at least reported.

We amended the text accordingly.

In general, it is possible that the RNA and protein of the Balbiani body would be different between different species. For example, Bucky ball, the only protein known to be essential for Balbiani body formation as found in zebrafish was reported to be rapidly evolving and does not have true homologs outside zebrafish and frogs (XVelo). I find it difficult to conclude a lack of Balbiani body in the mouse, based on the localization of these two specific proteins.

We completely agree with the reviewer on this point. Indeed, the conservation of intrinsically disordered proteins (IDPs) such as Velo1 and Buckyball are very poor in evolution (Boke et al., 2016; Bontems et al., 2009). We conclude that there is a lack of Balbiani body in mouse because of three main reasons:

1- Mouse primordial oocytes do not have any mitochondrial conglomeration (Figures 1G, S3B-E)
2- They do not have a proteinaceous matrix that might hold the contents of a Balbiani body (Figure 2, 3, S5 and S6).
3- Finally, the previous evidence suggesting two RNA binding proteins are localized to the Balbiani body cannot be repeated. In detail, the rationale behind the section regarding RAP55 and RNGTT is as follows: Localization of RAP55 and RNGTT proteins to the Golgi ring has been used as key evidence to claim the presence of a Balbiani body in mouse oocytes (Lei et al., 2020; Pepling et al., 2007). Here, we show that, when these experiments are performed quantitatively (in the case of RNGTT) or with appropriate antibody combinations (in the case of RAP55, please see above), we could not repeat these results (Figure 4). Therefore, in the absence of this key evidence, we concluded: “Thus, we conclude that the Golgi ring does not associate with RNGTT or RAP55, and does not necessarily host any RNA binding proteins.”

Fig. 5 - The images do not clearly represent the described text. In panel A bottom panel - 'Primary' seems to have no golgi ring.

A primary oocyte is an oocyte in the extensive growth phase (Adhikari and Liu, 2009; Eppig, 2001); thus, it would indeed not have a Golgi ring.

In panel C - the authors state examples of oocyte with and without golgi ring, but all panels seems to show one, except for the bottom row. This should be clarified or other representative images should be shown.

The reviewer is right to point out the antibody artefact in panel C (the green blob outside the oocyte). We replaced the panel with an additional image from the same replicate that does not contain any antibody artefacts.

E-F - this analysis is difficult to interpret as bulk data. The data has to be more specifically analyzed and presented. For example, it would be ideally performed by first scoring oocytes for FOXO nuclear localization, blindly to the golgi organization. Then the oocytes selected for either FOXO localization group should be scored for the presence of a golgi ring. The percentage of oocytes with or without golgi ring then should be presented for each FOXO localization category.

We are in the process of performing this additional analysis, and will add it to a supplementary figure, as asked by the reviewer.

This experiment is based on the claim that golgi ring disassembly by BFA disassembles the Balbiani

body, which remains controversial. It may still be possible that BFA does not affect the Balbiani body per se, making the results from this experiment difficult to interpret. It is also possible that the disruption of the golgi cause non-specific effect. For example if Balbiani body dissociation normally effects FOXO localization via a protein that requires a functional golgi organelle for its processing. More controls are needed to avoid misinterpretations.
We apologize if the rationale behind this experiment was not clear.

We completely agree with the reviewer that BFA does not affect a hypothetical Balbiani Body, and only dissolves the Golgi (ring). Indeed, as we wrote in the text:
"… we used Brefeldin A (BFA), one of the most specific compounds that acts on the Golgi apparatus to induce Golgi disassembly (Chardin and McCormick, 1999)".
The starting point of these experiments was our previous conclusion that the mouse does not have a Balbiani body. In fact, the previous section finished with the following paragraph:
"Since the Golgi ring does not associate with mitochondria, is not maintained within a proteinaceous matrix and does not co-localize with RNA-binding proteins, we conclude that the Golgi ring is not a marker for the Balbiani body in mouse primordial oocytes. Based on this, and together with our previous result showing that mouse oocytes lack mitochondrial conglomeration and an amyloid-like protein matrix, we conclude that mouse oocytes, unlike human and *Xenopus*, do not contain a Balbiani body."
We next asked whether the presence or absence of the Golgi ring is functionally related to oocyte dormancy, as it is present in primordial oocytes and dissociates upon oocyte activation. This is inspired by the correlation seen between the Golgi ring and dormancy previously in the literature (Lei et al., 2020; Lei and Spradling, 2016).

Panel B - The size and resolution of the images make it difficult to evaluate the formation of the golgi ring and FOXO localization. Images with increased size and resolution should be provided, as well as single channel images for each signal.

The images are now provided in Figure S7.

In Supp. Videos 3-6 the general brightness of signals seem to vary significantly. The authors should report on whether experimental conditions, including staining and image acquisition were kept uniform and consistent to allow for quantitative comparisons within and between experiments.

Experimental settings and image acquisition conditions were kept uniform and consistent through experiments. These ovaries were biological replicates extracted from different animals (but of the same litter [thus, replicate ovaries belong to 'sisters'] to try to avoid discrepancies between animals), and imaged whole-mount in confocal microscopes. Depending on the 3D-shape/ volume and lipid content of the ovary, one might expect differences in signal intensity (as these factors would affect the light path and thus, intensity of the signal). Having said this, the comparisons extracted from these images did not require fluorescent intensity measurements (we counted nuclear vs non-nuclear FOXO3A signal; as well as presence or absence of the Golgi ring); thus, the background fluorescence intensity levels in-between biological replicates is not a cause for concern.

Minor Comments

Comment 1:
Several statements in the introduction are not accurate, as follows:
"The earliest stage of a recognizable oocyte in the ovary is the primordial oocyte" - this is not correct. Oocyte differentiation initiates with the induction of meiosis, which occurs while oocytes continue to develop within a germline cyst. In mammals, the oocyte forms the primordial follicle only at pachytene stages of meiosis I, well within its differentiation. Similar consistent dynamics was also reported in zebrafish.

We have changed the text to ""The earliest stage of a recognizable follicle in the ovary is the primordial follicle, which contains a primordial oocyte".

"Primordial oocytes constitute the fixed ovarian reserve and are considered dormant as they do not grow nor divide" - this is not accurate. Primordial follicles develop to primary growing follicles and

a subset pool of primordial follicles are arrested as the ovarian reserve.

We rephrased the sentence to “The ovarian reserve consists of primordial oocytes, which are considered dormant as they do not grow nor divide"

"The Balbiani body is only present in early, dormant oocytes and dissociates upon oocyte activation." - This is not accurate. In Zebrafish the Blabiani body dissociates at late St. I of zebrafish oogenesis, well within primary follicle stages. In the mouse, the Balbiani body was observed in primary follicles in P7 postnatal ovaries at the latest. Moreover, in Zebrafish early Balbiani body formation was shown to begin at zygotene stages of meiosis I, while oocytes are still in the germline cyst (Elkouby et al., 2016). A similar early formation of the Balbiani body in early meiotic prophase (E14.5) germline cyst was shown in the mouse (Lei and Spradling, 2016). The oocyte is by no means 'dormant' at these stages. Altogether, stages that include the Balbiani body or its formation are not specific to primordial follicles.

In the literature, the Balbiani body is considered to be present in the earliest stages of oocytes: in stage I of zebrafish oogenesis (as the reviewer also commented), stage I of *Xenopus* oogenesis (Dumont, 1972) and ‘just before’ primordial follicle formation in mammals (see below) (Pepling et al., 2007). We would like to point out to the reviewer that, Pepling et al. also concluded that the Balbiani body is only observed in primordial follicles:

“The mouse Balbiani body forms from previously described mitochondrial aggregates just before primordial follicle formation and persists briefly in young primordial follicles. In growing follicles, mitochondria and ER disperse, and a well-defined Balbiani body is no longer found.”(Pepling et al., 2007)

In light of the reviewer’s concern that the Balbiani forms before the primordial follicle stage when oocytes are not yet dormant, we changed that sentence to;
“The Balbiani body is only present in early oocytes and dissociates upon oocyte activation."

Comment 2:

A better labeling of the species shown for panels in figures and cartoons would help orientation.

All done.

Reviewer #3 (Significance (Required)):

The subject of this investigation has potential for great interest by the communities of germ cell and developmental biologists, as well as cell biologists and reproduction biologists. However, unfortunately, I am afraid that with the concerns raised above, the data has to substantially improve before it can be relevant for this audience.

The authors report a conclusion that goes against several published reports. This off course would be completely appropriate, but in such case I would expect the authors to perform a more thorough investigation and present more solid data, which currently are not compelling to rule out the said previous reports. In one case, the authors report a finding that was already published by others.

I have expertise in Balbiani body biology

We thank the reviewer for thinking the subject of our investigation has potential for great interest. We would like to point out that our paper goes mainly against two research papers published by the same group; the Spradling lab. The first study is a contributed article in PNAS: (Pepling et al., 2007). The second study does not study aspects of the Balbiani body specifically but mostly concerns the germ cell cyst environment and transfer of organelles in between oocytes within the same nest in early mouse embryos:(Lei and Spradling, 2016). In fact, the first study suggests that until then, it was believed that a Balbiani body did not exist in mouse oocytes, however, none of the references they referred to for this statement include any statements about the presence or absence of a Balbiani body in mouse oocytes. Moreover, as we also detailed above in the chapter regarding RAP55,

Pepling et al., used two custom-made antibodies raised in the same species (rabbit) (Pepling et al., 2007; Wilhelm et al., 2005; Yang et al., 2006) to support their conclusions regarding colocalization with RAP55. Two primary antibodies raised in the same species are very likely to show spurious colocalization, which would explain the discordance between our and previous findings.
We apologized above for the statement referring to the panel in supplementary Figure S2, and have changed our wording to:
"We found that the Golgi ring had polarized Golgi stacks, and confirmed previous reports that it is associated with pericentrin"

4. Description of analyses that authors prefer not to carry out

Please include a point-by-point response explaining why some of the requested data or additional analyses might not be necessary or cannot be provided within the scope of a revision. This can be due to time or resource limitations or in case of disagreement about the necessity of such additional data given the scope of the study. Please leave empty if not applicable.

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Original submission

First decision letter

MS ID#: JOCES/2021/259394

MS TITLE: Comparative analysis of vertebrates reveals that mouse primordial oocytes do not contain a Balbiani body

AUTHORS: Laasya Dhandapani, Marion C Salzer, Juan M Duran, Gabriele Zaffagnini, Cristian De Guirior, Maria Angeles Martinez-Zamora, and Elvan Boke
ARTICLE TYPE: Research Article

Thank you for considering JCS with your reviews commons submission. I have gone through your MS and responses to the reviews questions and comments. I think your plan of action thoroughly addresses the most important issues they have raised. Consequently, I would be pleased to see a revised manuscript along the lines you have indicated. I would then make a quick decision.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.organd click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

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

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

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

First revision

Author response to reviewers' comments

We thank all the reviewers for their critical reading of the manuscript and excellent suggestions. **All three reviewers** expressed that the topic of our manuscript is **of high**

importance. **Two reviewers were mostly complimentary** in their comments and have suggested some specific experiments, and while more skeptical, a third reviewer has given constructive criticism and suggested several additional experiments. We have performed virtually all of these experiments and **the data they provide support and strengthen our conclusions**. We thank the reviewers for these suggestions, which strengthen our paper considerably. Below we detail specific reviewer comments and our responses. Major additions, new analyses, or changes that directly address reviewer comments are coloured in green in the main text. For other small changes, we did not track changes in this way.

Reviewer #1 (Evidence, reproducibility and clarity):

Laasya Dhandapani, et al have developed elegant methods for comparative characterization based on live imaging of primordial oocytes from Xenopus, mice and humans. Using specific markers for different intracellular organelles and pharmacological antagonists, they show that the three oocytes contain active mitochondria, Golgi apparatus and lysosomes. These organelles are included in a Balbiani body in Xenopus and human but not in mouse oocytes. In the latter they found in place of the Balbiani body a ring-shaped Gologi apparatus and the latter persisted when the oocytes were activated. The authors conclude that mouse oocytes do not contain a Balbiani body and that this structure is not associated with oocyte dormancy contradicting previous reports. This work is well done and interesting. However the fact that the oocyte of mice did not contain Balbiani body (Title) is not the main result of their work. This has already been shown in other species. It was shown from a long time that germ cells segregate from the soma early in development and that germ cell fate depends on the acquisition of a germ cell determinant commonly referred to as germplasma, an essential element of germ cell identity. In various animal species the subcellular composition of this germ plasm is different, and it has been shown that it contains various molecules such as RNA, proteins... Oocytes of certain species such as rats, hamsters, guinea pig, rabbits, nonhuman primates and humans have a Balbiani body similar in ultrastructure and behavior to those of Xenopus. But little information on their molecular composition and function is available. Mice oocytes on the contrary do not have any structure corresponding to the germ plasm, and no Balbiani body. The originality of this work is the comparative analysis of the subcellular composition of the oocytes of these 3 species. The authors show that all three oocytes contain mitochondria, Golgi apparatus, and that all organelles are functional. It was previously postulated that healthy mitochondria selectively associated with the Balbiani body to allow removal of defective mitochondrial genomes from ultimate germ cells.

We thank the reviewer for the positive comments and the encouragement. In light of their comments, we decided to change the title of our manuscript to:

“Comparative analysis of vertebrates reveals that mouse primordial oocytes do not contain a Balbiani body”.

Reviewer #1 (Significance (Required)):

The authors show that the ring-shaped Golgi apparatus in mice is not linked to a dormant state of the oocyte. It is known that the Balbiani body or related structure is characteristic of the formation of primordial germ cells which cannot develop when this structure is altered. However the link with a dormant state of the oocyte has not been the main opinion in the literature (see for example the recent reviews Chun So et al, Trends Cell Biology, 2021,31, 254-267 or Malgorzata Kloc et al, Current topics in Developmental Biology, 2004, 59, 1-36)..). However the authors do not study the apparition of these structures during development and the possible consequences of blocking different organelles on the subsequent development or function of the oocyte. It was previously hypothesized that the appearance and persistence of a Balbiani body was closely related to the type of the ovary and length of the oogenesis, and appears when oogenesis is rather slow but not in rapid oogenesis because the formation and transport of germplasm component occur rapidly (above References).

It is therefore an important elegant and detailed live-imaging study of germplasm composition in Xenopus, mice and humans which provides a true original knowledge. It should be noted that oocytes were isolated from neonatal mice, young adult Xenopus and from women aged 18

to 37 years. Further imaging and functional studies are needed to understand the function of the Balbiani body or the ring-shaped Golgi apparatus in germ cell growth, differentiation polarity and fertilization.

We agree with the reviewer that isolating primordial oocytes only from neonatal mice is not ideal for our analyses, considering that we compare them to young adult *Xenopus* and women. We initially preferred using neonatal mice because they are most commonly (in fact, almost exclusively these days) used in the literature to isolate primordial oocytes from mice (Castrillon et al., 2003; Eppig and Wigglesworth, 2000; Morohaku et al., 2016; Pepling et al., 2007; Shimamoto et al., 2019). However, we repeated our live-cell imaging experiments with primordial follicles isolated from young adult mouse (5-8 weeks) using markers for lysosomes, mitochondria and the Golgi apparatus. Primordial follicles isolated from adult mice also displayed active organelles, repeating similar patterns we observed in primordial follicles isolated from neonatal mice. We incorporated these results as a supplementary figure (Figure S3A-C), and inserted the following text:

"Primordial oocytes are almost exclusively obtained from neonatal mice in the recent literature (Shimamoto et al., 2019; Pepling et al., 2007; Morohaku et al., 2016; Castrillon et al., 2003; Eppig, et al., 2000). Since Xenopus and human oocytes in this study were isolated from young adults, we repeated our experiments using primordial oocytes obtained from young adult mice (5-8 weeks old). We found that similar to newly formed primordial oocytes in neonatal ovaries, primordial oocytes found in young adult ovaries also contained active mitochondria, lysosomes and Golgi apparatus. Thus, primordial oocytes isolated from neonatal and young adult mice are similar with regards to the activity of their organelles (Figure S3A-C)."

We thank the reviewer for bringing this up, which certainly improved our manuscript.

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

Asymmetric localization of cellular components is a shared feature of early oocytes. The Balbiani body is a nonmembrane bound compartment that has been observed in species ranging from insects to vertebrates, including mammals. This structure has been postulated to play a role in regulating oocyte dormancy, RNA localization and regulation, and sequestration of determinants, such as the germ plasm and patterning molecules in some animals, and allocation or sorting of cellular organelles, namely mitochondria. In recent years, the Balbiani body has been shown to assemble by a mechanism that involves intrinsically disordered proteins and amyloid formation. In this work the primary oocytes of three species, Xenopus, mouse, and human are characterized using live imaging, vital dyes, and inhibitors to probe the distribution and activity of cellular organelles. The live imaging approach that sets this work apart from previous studies examining cellular asymmetries in mouse, Xenopus, and human oocytes. Interestingly, this study revealed that lysosomes are active in vertebrate oocytes. In contrast, lysosomes are not thought to be active in the invertebrates examined thus far. The manuscript is well written and has several new and exciting findings, including a more detailed comparison of asymmetries and organelles within vertebrate oocytes that challenges the existing models for mammalian Balbiani body composition that were based largely on the mouse oocyte. Overall, the experiments appear to be well controlled, the data are of high quality, and the data provided largely support the conclusions, but could be strengthened by attending to some easily addressable matters, center around missing or unclear numbers for some of the experiments and that some of the analysis appears to have only been quantified in the mouse. Given that this is a comparative study, and that a key conclusion is that the mouse is different, the supporting quantitative data should be provided.

We thank the reviewer for these positive comments! We provide the quantitative data in this revised version, please see below.

Major:

One potential concern is that the human oocytes used were obtained from women who had undergone ovarian surgery. If the women had surgery because of cysts, cancer, or similar

reproductive health concerns, then it is possible that the oocytes may not represent are healthy normal oocytes. If this is a possibility, then this should be stated. It is probably a good idea to clarify even if it is not a concern - if these were all or in part from healthy women free of disease affecting the reproductive system.
We thank the reviewer for highlighting this, indeed we only used oocytes from women free of disease affecting the reproductive system. We now include the full study conditions, and the following statement in methods:

"Women fulfilling the inclusion criteria undergoing ovarian surgery were asked to participate in the study. Informed consent was obtained from all of them.
***Inclusion criteria**: Age between 18 to 35, fertile (assessed by un-induced menstrual cycles or presence of antral follicles identified by ultrasound examination), presence of at least one ovary, signed informed consent.*
***Exclusion criteria**: women with menopause, endometriosis, or who underwent bilateral oophorectomy.*

All oocytes incorporated in this study were from women free of disease affecting the reproductive system."

In Figure 1 panel C quantification of lysotracker is shown for mouse oocytes. Was similar analysis performed on the lysotracker labeled Xenopus and human oocytes? Including this analysis would provide a quantitative basis for comparison of these different oocytes.

We thank the reviewer for this suggestion; we now include Lysotracker intensity measurements for all three species, comparing puncta in oocytes and in the (somatic) follicle cells surrounding them (Figure 1B). We modified the main text accordingly to:

"The LysoTracker intensity was similar between somatic cells and primordial oocytes in all three species (Figure 1A, B), as well as between primordial and growing oocytes (GVs) in mouse (Figure S1C-D)."

Based on the TMRE staining shown in Figure 1H, the mouse and human oocytes appear similar. Both appear to have domains devoid of mitochondria? For the Xenopus and human oocyte analyses, how many oocytes were examined? From how many females? It would be helpful to include the specific quantification and criteria that were used to conclude that mitochondria distribution is different in human and mouse oocytes- Based on the examples shown, human looks quite different from Xenopus and more similar to the mouse oocyte. Is this a function of stage? The schematics in panel I are helpful, but please add labels above to indicate the species.

We thank the reviewer for bringing this up, which gives us a chance to clarify our discovery. First of all, at least three individuals were examined for each species; eight human, twenty-three mouse and nine *Xenopus* oocytes were counted for mitochondrial staining. We now include an aggregate information in the figure legend:

"Three individuals were examined for each species; at least 2 oocytes were imaged for each human, and at least 3 oocytes for each mouse and Xenopus"

The distribution of mitochondria in mouse and human oocytes looks, indeed, similar to each other and different from *Xenopus* at a first glance. However, a careful examination unraveled superficial similarities and highlighted features that are not obvious in the first place, mostly because of the major size differences of oocytes in these three species. Our examination concentrated on two basic points:

1. **Mitochondrial Conglomeration:** In human oocytes, the majority of the mitochondria is conglomerated adjacent to the nucleus, as in *Xenopus* oocytes. However, in mouse oocytes, mitochondria are distributed throughout the available space in the cytoplasm. In order to provide a quantification for this statement, **we now calculated the center of mitochondrial mass in *Xenopus*, mouse and human oocytes:** in mouse, the center of mitochondrial mass is located within the nucleus as mitochondria are

distributed throughout the cytoplasm. In humans and *Xenopus*, the center of mitochondrial mass remains within the Balbiani body or at the nucleus-Balbiani body border, as the majority of the mitochondria in these cells are conglomerated inside the Balbiani body. **We added this analysis as an additional supplementary figure (Figure S2B-C).**

2. **Not every cellular space not occupied by mitochondria is the same:** In mouse oocytes, we (and others) show that the distinct space in the cytoplasm that is not occupied by mitochondria is filled by the Golgi ring. In human oocytes, the "gap" in mitochondrial conglomeration has been observed numerous times in the literature, and corresponds to cytoplasmic membranes of unknown nature (Hertig, 1968; Hertig and Adams, 1967). **We do see this gap in mitochondrial staining in *Xenopus* oocytes too (Figure S2F) but the large size of these cells makes these gaps in mitochondrial staining look trivial. We now provide a side-by-side comparison of different sizes of oocytes and of this observed "gap" in mitochondrial staining for all three species (Figure S2F).** Our previous electron microscopy images in *Xenopus* (Boke et al., 2016), the literature in human oocytes (Baca and Zamboni, 1967; Hertig, 1968; Hertig and Adams, 1967) indicate that these membranes **do not** correspond to Golgi membranes which have very characteristic features. On the other hand, electron microscopy studies in mouse oocytes show that this gap is occupied with Golgi membranes (Lei and Spradling, 2016; Pepling et al., 2007). Finally, our recent immunofluorescence images of Golgi in human oocytes (Figure S3D) show that human oocytes do not contain a Golgi ring.

Thus, we conclude that although mouse and human oocytes look similar at first sight, these similarities are likely superficial.

It is not clear how many oocytes and individuals were examined for the proteostat experiments.

We apologise for this oversight. Three individuals were examined for each species; at least three oocytes were imaged for each human, ten oocytes for each mouse and five oocytes for each *Xenopus*. These numbers are now incorporated into the figure legend of the corresponding figure:

"Three individuals were examined for each species; at least 3 oocytes were imaged for each human, 10 oocytes for each mouse and 5 oocytes for each Xenopus."

Minor:

A general comment for the graphs in figures where oocytes of more than one species are shown, please include labels indicating which species is shown. If a graph or schematic contains data from more than one species, please add a key.

We added this information for all the figures that have oocytes from different species.

The title for Figure 2 concludes that mitochondria of mouse primordial oocytes are not maintained within a Balbiani body, but mitochondria are not analyzed in the figure. Please consider revising to reflect the data shown or include data on mitochondria.

We have changed the figure title to "Mouse primordial oocytes do not contain a large proteinaceous matrix".

Scale bars are missing in Figure 3A, for the enlarged panels and insets in Figure 4, Supp. Fig 1A top row, and Supp. 4A
We apologise for these oversights; scale bars are now added to all of the indicated figures.

In Supp. Fig 1C and Supp. Fig. 2 - assume the samples shown are mouse but this is not indicated.
We added the relevant species information to all figures.
Supp. Fig. 2C - please show each sample point sas in the other figures.

We have changed the format of the figure such that we show the data points.

Reviewer #2 (Significance (Required)):

Previous analysis of mouse primary oocytes identified a Golgi ring as the mouse Balbiani body. Based on the lack of mitochondria accumulation in this structure, it was suggested that the mammalian Balbiani body was unique from those of nonmammalian vertebrates. A striking finding from this work is that the mouse indeed appears to be unique but that somewhat unexpectedly, the primary oocytes of Xenopus and human appear to share similar cellular structures. Specifically, the distribution of organelles is different in mouse oocytes, and they appear to lack Balbiani bodies based on several criteria examined in this study: 1) no accumulation of mitochondria, 2) no evidence for RNA localization within a nonmembrane bound compartment, and 3) no evidence for an amyloid-like matrix. Moreover, staining and inhibitor treatments indicate that the Golgi ring has the hallmarks of a conventional Golgi structure and has no correlation to or instructive role in preventing or promoting oocyte activation, but does disassemble in response to oocyte activation. This work challenges the notion that mice even have a Balbiani body and provides evidence against the prevailing model that the Golgi ring regulates oocyte dormancy. The findings point to the importance of choosing a system to model a process based on a comparative cell biology approach rather than solely on evolutionary distance. The work is well-done, and the findings are exciting and significant to reproductive biologists investigating vertebrate oocyte biology, such as myself, and more broadly to cell biologists, developmental biologists, geneticists, and those investigating fertility and reproductive health

We thank the reviewer for these positive and encouraging comments.

Reviewer #3 (Evidence, reproducibility and clarity (Required)):
In this manuscript the authors aim to compare the content and organization of the Balbiani body between Xenopus, mouse and human oocytes. The Balbiani body is a conserved feature of oocytes across the animal kingdom. In frog and fish the Balbiani body is essential for oocyte polarity, proper fertilization and inheritance of maternal regulators that induces the dorsal-ventral axis of the future embryo, as well as its germline. In mouse, the Balbiani body has been associated with culling of successful oocytes during early formation of the primordial and primary follicle. Understanding the formation, organization and various features of the Balbiani body is therefore of great importance. While the Balbiani body has been extensively functionally studied in frog and fish, this aim is very important in mammals where functional tools for Balbiani body investigation are not yet available.

Based on vital dye labeling the authors examine the organization of the Balbiani body and conclude that mouse oocytes do not contain a Balbiani body. Unfortunately, I have substantial concerns regarding the experiments shown and I could not find the data compelling to support such a conclusion. Generally, the authors heavily rely on the use of vital dyes and do not employ complementing methodologies or approaches to support these findings. The rational of some of the experiments is not clear and rely on weak assumptions and some controls are missing. In addition, the number of oocytes/ovaries examined is rarely provided, and the presentation of the data is not always clear. I am detailing my comments below.

We understand that the reviewer expresses their concerns about the use of live-cell imaging dyes, as well as a perceived lack of analyses on fixed tissue samples. In the field, there are several studies performed on fixed ovary tissues (Castrillon et al., 2003; Kato et al., 2019; Lei and Spradling, 2016; Shimamoto et al., 2019). Although these studies have been pivotal in nature, and pushed the field forward in many ways, they have been limited by common problems associated with fixation: loss of information on organelle dynamics and activity; introduction of fixation and permeabilization artefacts such as the distortion of cell shape, hindering of epitopes that would be recognized by the antibody and the loss of membrane proteins. Moreover, a reliance on antibodies makes it very challenging to detect oocyte/germ cell-specific splice variants to mark organelles, and some species-specific proteins.

In fact, it is still impossible to find a non-sectioned or not fixed image or description of mammalian primordial oocytes in the literature. Our study advances on the previous work by employing live- cell imaging; the Reviewers 1 & 2 also highlighted our use of live-cell imaging as a significant strength. Only by live-cell imaging we can collect information regarding the activity of organelles and their dynamic distribution in the cytoplasm. However, in response to the reviewer's concern, we emphasize that we do not uniquely rely on live-cell imaging: our manuscript includes extensive immunofluorescence images with whole-ovary imaging (our preferred fixation method to avoid shape-distortions observed during fixation in large cells like oocytes) and in some cases, tissue sections (Figure 4, 5, S1F-G, S5A).

Moreover, in response to the reviewer's suggestions, we have added several more immunofluorescence images to strengthen our findings (Figure S3B, S3D-F). We thank the reviewer for helping us bridge the gap between live-cell imaging and fixed sections.

The authors conclude the "mouse oocytes do not contain a Balbiani body". As a constructive advice, I would like to comment that it is extremely challenging conceptually to prove that something does not exist. It may very well be that the mouse Balbiani body is different than that of other species in its content and/or organization, and I believe that the authors should consider this possibility.

We thank the reviewer for their comments. In our manuscript, we adopt the conventional and most widely used definition for the term Balbiani body: the name Balbiani body, or "mitochondrial cloud" as it was referred to as in older literature, is used to describe a membraneless super-organelle that clusters mitochondria (Banani et al., 2017; Bilinski et al., 2017; Bontems et al., 2009; Cox and Spradling, 2003; Guraya, 1979; Hertig, 1968; Hertig and Adams, 1967; Jamieson-Lucy and Mullins, 2019; Kloc et al., 2004; Marlow and Mullins, 2008; So et al., 2021; Van der Stricht, 1923). We therefore remain consistent with the literature in considering the presence of mitochondria within a proteinaceous matrix indicative as the defining characteristic of any structure that would be called "a Balbiani body". Because mouse oocytes lack mitochondrial conglomeration and do not contain a proteinaceous matrix indicative of a membraneless super-organelle -two defining characteristics of the Balbiani body- we conclude that mouse oocytes lack a Balbiani body. Furthermore, ours is not merely a negative finding; we positively identify the structures previously described as Balbiani bodies as in fact mis-identified Golgi rings: the structures formerly known as murine Balbiani bodies do not fulfill the criteria of a Balbiani body, as they associate neither with a proteinaceous matrix (Figure 2, 3 S5), nor mitochondria (Figure 2, S4A-B), nor with previously-published known RNA-binding proteins (Figure 4A-C). Therefore, our contribution is not only to "prove that something does not exist" but it is also to prove that existing structures have been misidentified.

Of course, there are epistemological limitations to any experiment: we cannot exclude the possibility that some currently unknown functions performed by the Balbiani body in humans and *Xenopus* may be performed by a structurally distinct organelle in mouse oocytes that does not fulfil any of the above criteria. However, this structure, then, would not be a Balbiani Body.

We would like to note that it is not so unusual for an organelle to be missing from a particular cell type. There are many examples, such as cells that lack centrosomes (Bornens, 2012; Schuh and Ellenberg, 2007) or mitochondria (Muller, 1988). Such cell types happen to contain types of centrosomal or mitochondrial material to organize their acentrosomal spindle assembly or electron transport function, respectively, but these are not assembled and so the cells lack the namesake organelles. In a similar way, we have shown that mouse oocytes do not contain a Balbiani body.

Major Comments

Fig. 1A - LysoTracker signal in the Xenopus oocyte looks more like background than a true signal. Also, the insert hides much of the shown oocyte, it is advisable to show the entire oocyte. Based on the images shown it is difficult to conclude that "vertebrate primordial oocytes have acidic, active lysosomes distributed in their cytoplasm" for the Xenopus oocyte.

Xenopus stage I oocytes are huge (250 microns), whereas lysosomes are very small (0,2-1,2 microns in diameter). Thus, we understand the reviewer's concern that the lysosomal puncta might come across as background staining without additional magnification.
We now address this concern by

1- including full-size, high-magnification images of *Xenopus* oocytes (see below, Figure R2 in the response to reviewers).
2- performing an additional experiment with Bafilomycin A1, a lysosome acidification inhibitor, to prove the specificity of the Lysotracker dye in *Xenopus* oocytes too. In the presence of Bafilomycin A1, lysosomes do not uptake Lysotracker and thus, do not show any specific Lysotracker staining (Figure S1B).

We thank the reviewer for their suggestion to make this conclusion stronger.

We also provide additional quantifications of lysosomal puncta inside the oocyte and in somatic cells surrounding the oocyte for all three species. The Lysotracker intensity in each follicle was similar between somatic cells and the oocyte of that follicle (Figure 1B), providing additional vigor for our conclusion that "vertebrate primordial oocytes have acidic, active lysosomes distributed in their cytoplasm"

Fig. 1B - It is unclear how primordial follicle oocytes are distinguished from somatic cells, some of which are almost the same size. Labelling with a specific marker (like Vasa for example) would help determining this. Also, only a portion of the GV oocyte is shown, which makes the evaluation of the findings difficult.
It is well-established to differentiate and classify oocytes according to their size and morphology in both mammalian and frog reproduction fields (Clarke, 2012; Dumont, 1972; Gougeon, 1986; Morohaku et al., 2016; Pedersen and Peters, 1968; Wang et al., 2017; Westergaard et al., 2007). Thus, we now included the size information for oocytes in the Materials and Methods section of the manuscript in the paragraph "Oocyte classification" and labelled primordial and growing oocytes in the figure. Moreover, since a DDX4 staining (Vasa homolog in mammals) would require fixation and would therefore not be compatible with LysoTracker staining, we show below (Figure R1) images of oocytes isolated from transgenic Stella-GFP mice (a germ cell marker) (Payer et al., 2006) labelled with LysoTracker, replicating our previous results and conclusions.

***NOTE**: We have removed unpublished data that had been provided for the referees in confidence.*

Fig. S2 - The authors state: "We found that the Golgi ring had polarized Golgi stacks, and associated with pericentrin", however, the localization of the golgi apparatus around Pericentrin has already been reported by Lei and Spradling, 2016

We apologise for this oversight; we have changed our wording to:
"We found that the Golgi ring had polarized Golgi stacks as it contained cis- and trans-Golgi markers (GM130 and TGN46 respectively) and confirmed previous reports that it is associated with pericentrin (Lei and Spradling, 2016)".

Fig. 1C - It should be described and demonstrated how puncta were selected and measured for this analysis.

We added this information as an additional supplementary file (please see Figure S1C) and expanded the materials and methods with detailed description.

Fig. 1G - The insert shown hides the image of the oocyte, it is advisable to show the entire oocyte, so that readers can evaluate the results.

We thank the reviewer for their suggestion, now we include the full oocyte and the magnified image as separate panels for Figure 1F (Figure 1G in our first submission). Moreover, we provide

all images of entire *Xenopus* oocytes in Figure R2 below for the reviewer without any insets. Unfortunately, for space concerns, we had to keep the insets in the main figures.

NOTE: We have removed unpublished data that had been provided for the referees in confidence.

Fig. 1 H - the authors conclude that the mitochondria localization in the mouse oocyte is different than in humans and frog. To this reviewer it seems that mouse and human oocytes have similar mitochondria localization pattern, and that Xenopus is apparent the exception.

We thank the reviewer for bringing this up, which gives us a chance to clarify our discovery. The distribution of mitochondria in mouse and human oocytes looks, indeed, similar to each other and different than *Xenopus* at a first glance. However, a careful examination unraveled superficial similarities and highlighted features that are not obvious in the first place, mostly because of the major size differences of oocytes in these three species. Our examination concentrated on two basic points:

1- **Mitochondrial Conglomeration:** In human oocytes, the majority of the mitochondria is conglomerated adjacent to the nucleus, as in *Xenopus* oocytes. However, in mouse oocytes, mitochondria are distributed throughout the available space in the cytoplasm. In order to provide a quantification for this statement, **we now calculated the center of mitochondrial mass in *Xenopus*, mouse and human oocytes: in mouse, the center of mitochondrial mass is located within the nucleus as mitochondria are distributed throughout the cytoplasm**. In humans and *Xenopus*, the center of mitochondrial mass remains within the Balbiani body or at the nucleus-Balbiani body border, as the majority of the mitochondria in these cells are conglomerated inside the Balbiani body. We added this analysis as an additional supplementary figure (Figure S2B-C).
2- **Not every cellular space not occupied by mitochondria is the same:** In mouse oocytes, we (and others) show that the distinct space in the cytoplasm that is not occupied by mitochondria is filled by the Golgi ring. In human oocytes, the "gap" in mitochondrial conglomeration has been observed numerous times in the literature, and corresponds to cytoplasmic membranes of unknown nature (Hertig, 1968; Hertig and Adams, 1967). **We do see this gap in mitochondrial staining all the time in *Xenopus* oocytes too (Figure S2F) but the large size of these cells make these gaps in mitochondrial staining look trivial. We now provide a side-by-side comparison of different sizes of oocytes and of this observed "gap" in mitochondrial staining for all three species (Figure S2F).** Our previous electron microscopy images in *Xenopus* (Boke et al., 2016) and the literature in human oocytes (Baca and Zamboni, 1967; Hertig, 1968; Hertig and Adams, 1967) indicate that these membranes **do not** correspond to Golgi membranes which have very characteristic features. On the other hand, electron microscopy studies in mouse oocytes show that this gap is occupied with Golgi membranes (Lei and Spradling, 2016; Pepling et al., 2007). Finally, our recent immunofluorescence images of Golgi in human oocytes (Figure S3D) show that human oocytes do not contain a Golgi ring.

Thus, we conclude that although mouse and human oocytes look similar at first sight, these similarities are likely superficial.

In entire figure 1, a use of Max Z projection seems less appropriate. A max projection will provide only the most intense signals in the oocyte. For a reliable evaluation of the distribution of the organelles shown, a Sum Z projection would recover all signals throughout the oocytes, allowing for better assessment of their distributions.

We provide sum-Z projections below in Figure R3. Upon preparing sum slices of the images (follicles are roughly ~25, 50 and 250 microns, in mouse, humans, and *Xenopus*, respectively) the signal within the primordial follicle was over-saturated in all 3 species. The resulting image was not clear and the organelle distribution could not be discerned especially in the

case of Lysotracker and NBD C_6-Ceramide probes, which are membrane-soluble and tend to accumulate weakly in all membranes. Hence, we prefer using max-Z projections in the manuscript.

***NOTE**: We have removed unpublished data that had been provided for the referees in confidence*.

In addition, the authors heavily rely on dyes for their analyses. Vital dyes can be very useful, but are limited in tissue penetration for example, which could result in varying signal intensities between different parts of the tissue and between experiments. Validation by other methods, such as ultrastructure analysis and immune-fluorescent staining, or transgenically expressed or injected markers (for mouse and Xenopus, respectively) is required to support their conclusion.

We thank the reviewer for their concern. We provide below diffusion calculations for biological systems, considering the size of oocytes, and their attached somatic cells (pasted below):

NOTE: We have removed unpublished data that had been provided for the referees in confidence.

Here were used the equation for one-dimensional mean square displacement of a live-cell imaging probe (Berg, 2018) assuming lipid-like property and spherical symmetry of the oocyte to obtain the time for diffusion of the probe (t) as:

$$t = \frac{r^2}{2D}$$

Where r is the radius of the somatic cell and oocyte that comprise the follicle, D is the diffusion constant which was assumed to be 5μm^2/sec for a lipid diffusing through the membrane and 50 μm^2/sec for a lipid diffusing through cytoplasm. The total diffusion time is calculated as:

$$t_{total} = t_{PM1} + t_{somatic\ cell\ cytoplasm} + t_{PM2} + t_{OPM} + t_{oocyte\ cytoplasm}$$

Where PM1 represents the somatic cell plasma membrane facing the medium in which the dye is dissolved, PM2 is the plasma membrane of the somatic cell in contact with the oocyte and OPM is oocyte plasma membrane. An example for the diffusion through a mouse follicle is shown:

NOTE: We have removed unpublished data that had been provided for the referees in confidence.

As the reviewer can see, even in the case of large *Xenopus* oocytes, we are not limited by diffusion (Table 1). Moreover, all our labelling experiments provided similar or higher intensity of fluorescence inside oocytes compared to that of the surrounding somatic cells, which would go against limited penetration.

Finally, as suggested by the reviewer, we now provide in a supplementary figure (Figure S3D-F) immunofluorescence images of primordial oocytes performed in fixed tissue sections of the three vertebrates supporting our findings using live-imaging regarding the distribution of organelles.

Fig.2 - Based on the images shown, it is difficult to assess and compare the signal of the dye used, which shows substantial background. To my eyes, the data shown for mouse looks similar to that of the human oocyte. In addition, it is unclear how consistent and quantitative are the

staining and imaging conditions that are used. The Xenopus image for example looks generally much brighter than the other species' oocytes.
In addition, co-labeling for a landmark for the Balbiani body (like Pericentrin, golgi etc.,) would help to determine whether the shown signals originate from the Balbiani body versus the remaining cytoplasm.
This data as provided here does not support the conclusion that mouse Balbiani bodies do or do not contain amyloid structures. In addition to the dye used, ultrastructure analysis is required. The authors also previously reported the use of a vital dye called ThT for identifying amyloid structures in live oocytes. It would be good to validate the data with such dye which also would help circumvent fixation artefacts.

"N"s are not provided for almost all experiments.

We understand the reviewer's concern, and now provide a thresholding analysis on the images that clearly shows that human and *Xenopus* oocytes have localized Proteostat staining in their cytoplasm (top 10% fluorescent intensity thresholding masks applied), whereas mouse oocytes do not (Figure S4A). In fact, we only observe nucleoli distinctly in mouse oocytes, which serve as a positive control (as nucleoli are phase separated compartments and shown to contain amyloid- like material in some species, or under certain circumstances (Alberti and Carra, 2019; Frottin et al., 2019; Hayes and Weeks, 2016). Thresholding is an objective criterion (as opposed to what is visible to an eye), and clearly shows that *Xenopus* and human oocytes have cytoplasmic Proteostat-positive assemblies in the shape of the Balbiani body, whereas mouse oocytes only have punctae distributed in their cytoplasm. We also incorporate zoomed out images from mouse and human ovaries that make the proteostat staining of Balbiani bodies (or lack-there-of) more apparent (Figure S4B).

In order to determine whether the Proteostat signal in human and *Xenopus* oocytes originates from the Balbiani body, we performed co-staining of Proteostat with the mitochondrial marker citrate synthase. This staining clearly shows that the Proteostat signal overlaps with that of citrate synthase in both species, thus confirming our conclusions. In mouse oocytes, the Proteostat staining in the cytoplasm barely exceeds background fluorescence levels despite its overlap with some mitochondria.
Unfortunately, citrate synthase immunohistochemistry is incompatible with the manufacturer's protocol used for Proteostat staining. We thus followed the IHC protocol and added Proteostat to the secondary antibody. While we were able to detect Proteostat under these conditions, the signal was a lot weaker and overall less reliable when compared to the manufacturer's protocol even for human and *Xenopus* sections (see Figure R4 below). Therefore, we added these images here for the reviewer's discretion, however, we would not like to include them in the main manuscript file.

NOTE: We have removed unpublished data that had been provided for the referees in confidence.

Ultrastructure analyses were already performed in the literature for all three vertebrate oocytes (please see below). The available data is of high quality and support our conclusions:

- In humans, apart from a mitochondrial conglomeration (Baca and Zamboni, 1967; Hertig, 1968; Hertig and Adams, 1967), "fibrils" were noted in the Balbiani body: "The prominent center, measuring up to 4.5 μm in diameter (…) is composed of electron opaque deposits embedded within a matrix of fine fibrils. These dense granules may become periodically aligned to form radiating fibrils which apparently merge with peripheral coarse fibers measuring up to 0.13 μm in diameter." (Hertig, 1968)
- In *Xenopus*, we and others showed mitochondrial conglomeration in oocyte cytoplasm without a surrounding membrane (Boke et al., 2016; Kloc et al., 2004).
- In mouse, Pepling et al., and others observed **a diffuse mitochondrial distribution** in the cytoplasm and a Golgi ring (Pepling et al., 2007; Wischnitzer, 1967; Wischnitzer, 1970), different than frog and human oocytes.

We apologize for not including N numbers; they are now included in the figure legends.

Unfortunately, due to the effect of Covid19 pandemic on elective surgeries, it is currently very difficult to receive human ovary samples and thus, we cannot provide live imaging of human oocytes with ThT. However, we include below (Figure R4) live ThT staining of mouse primordial oocytes and frog oocytes with ThT. *Xenopus* oocytes have a ThT positive conglomeration in their cytoplasm (the Balbiani body), whereas mouse oocytes only have diffuse ThT staining, even with a 10X higher ThT concentration than we used for *Xenopus* oocytes.

NOTE: We have removed unpublished data that had been provided for the referees in confidence.

Fig. 3 - The observation that mitochondria can localize to the position of the golgi is not convincing to conclude the lack of a Balbiani body or a proteinaceous structure. This experiment relies on three assumptions: 1. Proteins and organelles cannot be found closely adjacent to one another. However, in the Balbiani body of zebrafish and Xenopus, organelles are embedded within or surrounded by RNA-protein (RNP) granules. At the confocal resolution shown, one cannot conclude the lack of protein complexes that could still be present around the 'invading' mitochondria. 2. The golgi apparatus within a Balbiani body is not associated with such RNP granule. This was not ruled out. If this is the case, then dissociation of the golgi might also affect the structure of the Balbiani body and result with redistribution of mitochondria. 3. The mitochondria are not part of the Balbiani body. This was not proven. Balbiani body RNP can be associated with the 'invading' mitochondria, and in this case the results would not conclude a lack of Balbiani body. In fact, the RNP complexes that are found in piRNA granules in germ cells across vertebrate ad invertebrate species were described as nuage, electron-dense material (presumptive RNPs) that is associated with mitochondria. This is consistent with observations of intermingled RNP and mitochondria (and other organelles) in the Balbiani body in fish and frog.

In addition, no controls are provided to rule out non-specific effects in this experiment. In the measurement of the area occupied by mitochondria, the authors should specify whether this analysis was performed on single optical sections, or on Z projections. Altogether, I am afraid that the rational and technical aspects of this experiment are not compelling to conclude a lack of a Balbiani body.

We thank the reviewer for giving us an opportunity to clarify the rationale behind this experiment. The only assumption we rely on is that the compounds we use to dissociate the Golgi apparatus do not act on the hypothetical protein matrix. We believe that this assumption is correct for 2 reasons: First, we have used two compounds with two completely different modes of action to dissociate the Golgi apparatus: BFA, a compound long hailed for its specificity to act on the Golgi apparatus (Chardin and McCormick, 1999) and Nocodazole, a microtubule drug that is widely used in the literature to dissociate the Golgi (Cole et al., 1996; Turner and Tartakoff, 1989). Second, BFA does not have any effect on the Balbiani body in *Xenopus* oocytes (as shown in Figure 1C). An elegant study already showed that Nocodazole (for that matter, complete disruption of microtubules) does not have any effect on the structure of the *Xenopus* Balbiani body (Chang et al., 2004).

To specifically answer the reviewer's points above:

1- Proteins and organelles cannot be found closely adjacent to one another. However, in the Balbiani body of zebrafish and Xenopus, organelles are embedded within or surrounded by RNA-protein (RNP) granules. At the confocal resolution shown, one cannot conclude the lack of protein complexes that could still be present around the 'invading' mitochondria.

We did not assume organelles cannot be adjacent to each other. On the contrary, the mitochondrial exclusion zone (MEZ) calculations in this very figure rely on organelles moving and mixing (in case of mitochondria, through fusion and fission) with each other in the available cytoplasm to fill the place generated by the dissociated Golgi apparatus.

2- The golgi apparatus within a Balbiani body is not associated with such RNP granule. This was not ruled out. If this is the case, then dissociation of the golgi might also affect the structure of the Balbiani body and result with redistribution of mitochondria

It is certainly a possibility, that the Golgi apparatus is associated with an RNP granule; especially considering proteins like Trailer hitch, the RAP55 orthologue in *Drosophila*, resides at ER-exit sites (Wilhelm et al., 2005), where the Golgi apparatus tends to be. However, biomolecular condensates are de-mixed from the surrounding cytoplasm, and in case of the Balbiani body, they have a solid or amyloid-like nature (Banani et al., 2017; So et al., 2021; Woodruff et al., 2018). Thus, a large proteinaceous matrix should be present where the Balbiani body is. Therefore, two pieces of evidence go against the likelihood of the statement above:

1. The Proteostat did not detect any phase-separated compartment in mouse oocytes, whereas it clearly marked the Balbiani body of human and X*enopus* oocytes (Figure 2, S4). Thus, it is highly unlikely that there is a proteinaceous matrix associated with the Golgi ring in mouse oocytes.
2. A large proteinaceous phase-separated compartment is unlikely to be dissociated by the dissociation of the Golgi apparatus it has been associated with. This certainly has not been observed before in the literature. Supporting evidence for this statement is that we already showed in our manuscript that the *Xenopus* Balbiani body is not affected by dissociation of the Golgi apparatus (Figure 1F).

3- The mitochondria are not part of the Balbiani body. This was not proven. Balbiani body RNP can be associated with the 'invading' mitochondria, and in this case the results would not conclude a lack of Balbiani body.

We have shown in Figure 3 that mitochondria are not associated with the Golgi ring, and thus, are not part of a Balbiani-like compartment in oocytes. Considering the definition of a Balbiani body, or mitochondrial cloud as it has been referred to earlier, always include conglomeration of mitochondria, not having a mitochondrial conglomeration (irrespective of its association with the Golgi ring), is enough to conclude that mouse oocytes do not have a Balbiani body.

In the measurement of the area occupied by mitochondria, the authors should specify whether this analysis was performed on single optical sections, or on Z projections.
The MEZ calculations were performed on the equatorial sections of the oocytes.

Fig. 4 - In the references mentioned, I could not find data showing the localization of RNGTT in the Balbiani body.

We apologise for this oversight and now include the reference to the preprint regarding RNGTT localisation to the mouse Balbiani body (Lei et al., 2020).

RAP55 was shown to localize to the Balbiani body in the mouse, and it is possible that it represents a golgi marker and not necessarily a Balabiani body marker. However, to rule out previous findings from Pepling et al., 2007, I would expect more thorough investigation, attempting to test RAP55 localization at different oocyte stages. Pepling et al, showed data from E14.5, PND1, PND3. To conclude that RAP55 is completely absent from the Balbiani body, would require examination at different stages in order to rule our transient localization.

As suggested by the reviewer, we performed wholemount immunofluorescence analysis of ovaries from embryonic mice (embryonic day 14.5, E14.5), 1 day after birth (PND1) and 4 days after birth (PND4) using antibodies against the RNA-binding protein RAP55 and GM130 to detect the Golgi ring (Figure 4C). We would like to note that Pepling et al. did not show such co-staining of RAP55 and GM130 (Pepling et al., 2007, PNAS). At E14.5, we detected weak staining for RAP55 in primordial oocytes (Figure 4C), in accordance with the findings of Pepling et al. At PND1 and PND4, the expression of RAP55 was oocyte specific and localized to cytoplasmic granules, again in accordance with Pepling et al. However, RAP55 granules were excluded from the Golgi ring at both stages (Figure 4C, blue arrowheads). We thus conclude that throughout oocyte formation RAP55 is completely absent from the Golgi ring.

We would like to note that Pepling et al., 2007 used two custom-made antibodies raised in the same species (rabbit) (Pepling et al., 2007; Wilhelm et al., 2005; Yang et al., 2006) to support their conclusions regarding co-localisation of Trailer hitch and RAP55, and have not shown any evidence regarding RAP55 localisation to the Golgi ring. Two primary antibodies raised in the same species are very likely to show spurious co-localisation, which would explain the discordance between our and previous findings.

It should be noted whether single sections or z projections are shown, and the entire volume of the golgi/Balbiani body regions, as well as this of the entire cytoplasm should be shown, or at least reported.

We amended the text accordingly.

In general, it is possible that the RNA and protein of the Balbiani body would be different between different species. For example, Bucky ball, the only protein known to be essential for Balbiani body formation as found in zebrafish was reported to be rapidly evolving and does not have true homologs outside zebrafish and frogs (XVelo). I find it difficult to conclude a lack of Balbiani body in the mouse, based on the localization of these two specific proteins.

We completely agree with the reviewer on this point. Indeed, the conservation of intrinsically disordered proteins (IDPs) such as Velo1 and Buckyball are very poor in evolution (Boke et al., 2016; Bontems et al., 2009). We conclude that there is a lack of Balbiani body in mouse because of three main reasons:

1. Mouse primordial oocytes do not have any mitochondrial conglomeration (Figures 1G, S2B-E)

2. They do not have a proteinaceous matrix that might hold the contents of a Balbiani body (Figure 2C-D, 3C-F and S4C).

3. Finally, the previous evidence suggesting two RNA binding proteins are localized to the Balbiani body cannot be repeated. In detail, the rationale behind the section regarding RAP55 and RNGTT is as follows: Localization of RAP55 and RNGTT proteins to the Golgi ring has been used as key evidence to claim the presence of a Balbiani body in mouse oocytes (Lei et al., 2020; Pepling et al., 2007). Here, we show that, when these experiments are performed quantitatively (in the case of RNGTT) or with appropriate antibody combinations (in the case of RAP55, please see above), we could not repeat these results (Figure 4A-C). Therefore, in the absence of this key evidence, we concluded: "*Thus, we conclude that the Golgi ring does not associate with RNGTT or RAP55, and does not necessarily host any RNA binding proteins.*"

Fig. 5 - The images do not clearly represent the described text. In panel A bottom panel - 'Primary' seems to have no golgi ring.

We thank the reviewer for their comment. The primary oocyte is an oocyte in the extensive growth phase (Adhikari and Liu, 2009; Eppig, 2001); thus, it would not have a Golgi ring.

In panel C - the authors state examples of oocyte with and without golgi ring, but all panels seems to show one, except for the bottom row. This should be clarified or other representative images should be shown.

The reviewer is right to point out the antibody artefact in panel C (the green blob outside the oocyte). We replaced the panel with an additional image from the same replicate that does not contain any antibody artefacts.

E-F - this analysis is difficult to interpret as bulk data. The data has to be more specifically analyzed and presented. For example, it would be ideally performed by first scoring oocytes for FOXO nuclear localization, blindly to the golgi organization. Then the oocytes selected for either FOXO localization group should be scored for the presence of a golgi ring. The

percentage of oocytes with or without golgi ring then should be presented for each FOXO localization category.

We thank the reviewer for this useful suggestion. We had already scored the oocytes for FOXO3A nuclear localization irrespective of Golgi organization and this result was presented in Figure 5D of the submitted manuscript. We have now analysed the data as suggested by the reviewer and also show the Golgi ring localization in oocytes (only) with cytoplasmic FOXO staining (Fig 5F).
Also, we included the overall number of oocytes with and without the Golgi ring, and their FOXO3 staining status in supplementary figure 5B. A statistical analysis reveals an R-squared (R^2) value of 9 (e-05) that indicates the presence of a Golgi ring and nuclear FOXO3 localisation are not dependent variables, further supporting our claims. We thank the reviewer again for their
suggestion to make this conclusion easier to understand for the untrained eyes.

This experiment is based on the claim that golgi ring disassembly by BFA disassembles the Balbiani body, which remains controversial. It may still be possible that BFA does not affect the Balbiani body per se, making the results from this experiment difficult to interpret. It is also possible that the disruption of the golgi cause non-specific effect. For example if Balbiani body dissociation normally effects FOXO localization via a protein that requires a functional golgi organelle for its processing. More controls are needed to avoid misinterpretations.

We apologize if the rationale behind this experiment was not clear.

We completely agree with the reviewer that BFA does not affect a hypothetical Balbiani Body, and only dissolves the Golgi (ring). Indeed, as we wrote in the text:
"… we used Brefeldin A (BFA), one of the most specific compounds that acts on the Golgi apparatus to induce Golgi disassembly (Chardin and McCormick, 1999)".
The starting point of these experiments was our previous conclusion that the mouse does not have a Balbiani body. In fact, the previous section finished with the following paragraph:
"Since the Golgi ring does not associate with mitochondria, is not maintained within a proteinaceous matrix and does not co-localize with RNA-binding proteins, we conclude that the Golgi ring is not a marker for the Balbiani body in mouse primordial oocytes. Based on this, and together with our previous result showing that mouse oocytes lack mitochondrial conglomeration and an amyloid-like protein matrix, we conclude that mouse oocytes, unlike human and *Xenopus*, do not contain a Balbiani body."
We next asked whether the presence or absence of the Golgi ring is functionally related to oocyte dormancy, as it is present in primordial oocytes and dissociates upon oocyte activation. This is inspired by the correlation seen between the Golgi ring and dormancy previously in the literature (Lei et al., 2020; Lei and Spradling, 2016).

Panel B - The size and resolution of the images make it difficult to evaluate the formation of the golgi ring and FOXO localization. Images with increased size and resolution should be provided, as well as single channel images for each signal.

The single channel images are now provided in Figure S5 with increased size.

In Supp. Videos 3-6 the general brightness of signals seem to vary significantly. The authors should report on whether experimental conditions, including staining and image acquisition were kept uniform and consistent to allow for quantitative comparisons within and between experiments.

Experimental settings and image acquisition conditions were kept uniform and consistent through experiments. These ovaries were biological replicates extracted from different animals (but of the same litter [thus, replicate ovaries belong to 'sisters'] to try to avoid discrepancies between animals), and imaged whole-mount in confocal microscopes. Depending on the 3D-shape/ volume and lipid content of the ovary, one might expect differences in signal intensity (as these factors would affect the light path and thus, intensity of the signal). Having said this, the comparisons extracted from these images did not require fluorescent intensity

measurements (we counted nuclear vs non-nuclear FOXO3A signal; as well as presence or absence of the Golgi ring); thus, the background fluorescence intensity levels in-between biological replicates is not a cause for concern.

Minor Comments

Comment 1:
Several statements in the introduction are not accurate, as follows:
"The earliest stage of a recognizable oocyte in the ovary is the primordial oocyte" - this is not correct. Oocyte differentiation initiates with the induction of meiosis, which occurs while oocytes continue to develop within a germline cyst. In mammals, the oocyte forms the primordial follicle only at pachytene stages of meiosis I, well within its differentiation. Similar consistent dynamics was also reported in zebrafish.

We have changed the text to "The earliest stage of a recognizable follicle in the ovary is the primordial follicle, which contains a primordial oocyte".

"Primordial oocytes constitute the fixed ovarian reserve and are considered dormant as they do not grow nor divide" - this is not accurate. Primordial follicles develop to primary growing follicles and a subset pool of primordial follicles are arrested as the ovarian reserve.

We rephrased the sentence to “The ovarian reserve consists of primordial oocytes, which are considered dormant as they do not grow nor divide"

"The Balbiani body is only present in early, dormant oocytes and dissociates upon oocyte activation." - This is not accurate. In Zebrafish the Blabiani body dissociates at late St. I of zebrafish oogenesis, well within primary follicle stages. In the mouse, the Balbiani body was observed in primary follicles in P7 postnatal ovaries at the latest. Moreover, in Zebrafish early Balbiani body formation was shown to begin at zygotene stages of meiosis I, while oocytes are still in the germline cyst (Elkouby et al., 2016). A similar early formation of the Balbiani body in early meiotic prophase (E14.5) germline cyst was shown in the mouse (Lei and Spradling, 2016). The oocyte is by no means 'dormant' at these stages. Altogether, stages that include the Balbiani body or its formation are not specific to primordial follicles.

In the literature, the Balbiani body is considered to be present in the earliest stages of oocytes: in stage I of zebrafish oogenesis (as the reviewer also commented), stage I of *Xenopus* oogenesis (Dumont, 1972) and ‘just before’ primordial follicle formation in mouse (see below) (Pepling et al., 2007). We would like to point out to the reviewer that, Pepling et al. also concluded that the Balbiani body is only observed in primordial follicles:

“The mouse Balbiani body forms from previously described mitochondrial aggregates just before primordial follicle formation and persists briefly in young primordial follicles. In growing follicles, mitochondria and ER disperse, and a well-defined Balbiani body is no longer found.”(Pepling et al., 2007)

In light of the reviewer’s concern that the Balbiani forms before the primordial follicle stage when oocytes are not yet dormant, we changed that sentence to;
“The Balbiani body is only present in early oocytes and dissociates upon oocyte activation."

Comment 2:

A better labeling of the species shown for panels in figures and cartoons would help orientation.

We thank the reviewer for their suggestion. We have now incorporated labels for the species shown in the panels of the figures where it is required.

Reviewer #3 (Significance (Required)):

The subject of this investigation has potential for great interest by the communities of germ

cell and developmental biologists, as well as cell biologists and reproduction biologists. However, unfortunately, I am afraid that with the concerns raised above, the data has to substantially improve before it can be relevant for this audience.

The authors report a conclusion that goes against several published reports. This off course would be completely appropriate, but in such case I would expect the authors to perform a more thorough investigation and present more solid data, which currently are not compelling to rule out the said previous reports. In one case, the authors report a finding that was already published by others.

I have expertise in Balbiani body biology

We thank the reviewer for thinking the subject of our investigation has potential for great interest. We would like to point out that our paper goes mainly against two research papers published by the same group; the Spradling lab. The first study is a contributed article in PNAS from 2007, when contributed articles did not go through peer-review process: (Pepling et al., 2007). The second study does not study aspects of the Balbiani body specifically but mostly concerns the germ cell cyst environment and transfer of organelles in between oocytes within the same nest in early mouse embryos:(Lei and Spradling, 2016). In fact, the first study suggests that until then, it was believed that a Balbiani body did not exist in mouse oocytes, however, none of the references they referred to for this statement include any statements about the presence or absence of a Balbiani body in mouse oocytes. Moreover, as we also detailed above in the chapter regarding RAP55, Pepling et al., used two custom-made antibodies raised in the same species (rabbit) (Pepling et al., 2007; Wilhelm et al., 2005; Yang et al., 2006) to support their conclusions regarding colocalization with RAP55. Two primary antibodies raised in the same species are very likely to show spurious colocalization, which would explain the discordance between our and previous findings.

We once again apologize for the statement referring to the panel in supplementary Figure S2, and have changed our wording to:
"*We found that the Golgi ring had polarized Golgi stacks as it contained cis- and trans-Golgi markers (GM130 and TGN46 respectively) and confirmed previous reports that it is associated with pericentrin (Lei and Spradling, 2016)*"

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Second decision letter

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MS TITLE: Comparative analysis of vertebrates reveals that mouse primordial oocytes do not contain a Balbiani body

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ARTICLE TYPE: Research Article

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