

Increased DNA damage in full-grown oocytes is correlated with diminished autophagy activation

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

General comments:

1. In this study, the authors propose that oocyte failure to repair DNA damage is due to the inability of repair proteins to access closed chromatin and that this is due to lack of autophagy induction. I did not find that there was sufficient evidence to support this conclusion. Although there are some tantalising observations, these observations could be coincidental and the data as presented do not convincingly demonstrate that DNA repair, chromatin configuration and autophagy are directly connected.
2. It is stated in the abstract that DNA repair is not robust in mammalian oocytes. This claim is too broad as there is good evidence that immature oocytes have very robust DNA repair. The abstract should be specific about what stage oocytes are being referred to (i.e. fully grown). Furthermore, contrary to the major premise of this paper that fully grown oocytes are deficient in DAN repair, there is evidence that fully-grown meiotically arrested GVs oocytes can repair DNA damage (e.g. Collins 2015 and Cai 2023).
3. The introduction needs to more clearly explain in detail what is known about the role of autophagy, chromatin configuration and in the repair of DNA damage.
4. As summarised in Figure 4 (schematic, top panel), and inferred by the focus of the introduction, the authors are proposing that age-associated aneuploidy is caused by DNA double strand breaks, leading to failure of chromosomes to segregate properly at meiosis 1. Conceptionally, it is not clear how DNA fragmentation caused by DNA dsDNA breaks would result in lagging chromosomes and chromosomal aneuploidies (like trisomy 21). In the schematic, they also show breaks in both chromatids of a bivalent- was this intentional, as it implies the break occurred before DNA replication (which literature suggests would be efficiently repaired during prophase arrest, long before maternal aging occurs)?

Specific comments

1. Line 98-99- it's not really unexpected that oocytes have differently to somatic cells. Oocytes do a lot of things differently to somatic cells. Suggest revising wording.
2. What is the direct evidence that it's the failure to activate autophagy that causes altered chromatin structure and failure of RAD51 to localise to sites of DNA damage, failed DNA repair and increased aneuploidy?
3. Figure 1c- how were foci counted- H2AX is not distinct foci I these images?
4. Figure 1 indicates that etoposide treated oocytes had twice the number of foci as controls, with a cluster of oocytes having the same number of foci as controls. Following on from this, it is stated that 40% of the oocytes progressed to MII- were these just the oocytes that had similar H2AX to controls?
5. The legend for Figure 1 E-K indicates that the oocytes were released from arrest and then allowed to progress through to MII with no period of recovery. It seems very unlikely that chromosomes could segregate properly if there are up to 150 DSBs that have not been given time to repair- and yet only a single lagging chromosome is seen, rather than DNA fragments scattered all over the place, as reported by others. How can this be explained?
6. Figure 1 I-K. It is not clear what is meant by "compact" chromosome morphology? I agree the DNA looks different after DNA damage, but not compact. Indeed, the DNA looks more diffuse in the treated oocytes. The phenotypes of UV and IR exposed DNA do not look the same as etoposide, and I'm not really sure what the authors are getting at here because of course the morphology of damaged and fragmented DNA is different to intact DNA.
7. Fig 1 I-K. Did the authors really expose the oocytes to 100Gy IR? This is an extraordinarily high dose.
8. Fig 1 L-N- this is contrary to other reports showing that fully grown GVs can repair high levels of etoposide induced DNA damage (e.g. Collins 2015, Cai et al 2023).

9. Fig 1G and H are not referred to in the text.
10. 40% of oocytes progress to MII, how many to MI?
11. Rapamycin is an inhibitor of the mTOR pathway, which has diverse roles. It is not specific to autophagy induction. The rationale for using rapamycin to rescue DNA damage is not clearly explained in the text. The authors frequently refer to autophagy induction by rapamycin leading to a certain result or outcome, but its not possible to say if the autophagy or some other aspect of mTOR inhibition. E.g. it is concluded that "inefficient autophagy activation contributes to closed chromatin configuration in DNA damage oocytes" line 315-316. But only a handful of experiments (Figure 2E-G) in this paper were conducted with autophagy inhibitor (spautin-1), thus many of the observed rapamycin effects cannot be directly linked to autophagy at this stage.
12. The loss and induction of LC3II does not look very convincing in Figure 2E. How many oocytes and how many replicates?
13. Figure 2 A/B- Why is the autophagy activity reduced after DNA damage?
14. Line 198- weakened DDR as compared to what?
15. Figure 2B is missing an etop + rapamycin group.
16. Ref 36 is a conference abstract.
17. What is Nir in Figure 3G and H? This is not explained in the figure legend.
18. Line 254- It was stated that inducing autophagy (exposing to rapamycin) before inducing DNA damage reduces H2AX foci, and it was then concluded that autophagy reduces DNA damage. How do the authors know that rapamycin didn't interfere with the phosphorylation of histone H3 rather than actually reducing DNA breaks? Were comet assays performed to confirm this? Additionally, Spautin-1 should be used to confirm that this phenomenon is autophagy related. Similarly, although rapamycin reduced pATM levels, this could be unrelated to autophagy.
19. Line 275-282 (Figure 3). Elevated levels of RAD51 were not present after exposure to etoposide or UV. This is probably because there is a switch from dependence on HR to NHEJ in fully grown oocytes. The authors should also use a marker of NHEJ repair. Interestingly Rapamycin did increase RAD51 foci (rescue is not the right term in this instance), possibly due to impairment of the switch to NHEJ (notably, in Rapamycin can actually suppress both HR and NHEJ). Whether this is related to autophagy in oocytes is not known and cannot be determined from the data as presented.
20. Experiments were done to demonstrate that oocytes might have a more closed chromatin structure after damage (and this this structure could be opened with Rapamycin). But whether this closed structure means that DNA repair proteins can not access DNA breaks is not clear.

Reviewer #2

(Remarks to the Author)

In their manuscript "Diminished autophagy activation is a crucial factor for weakened DNA damage repair in full-grown oocytes" Sun et al. claim that aneuploidy in oocytes is linked to reduced autophagy induction following DNA damage. Upon etoposide treatment, they observe gH2AX induction that they could reduce by rapamycin treatment. The central argument of the paper is the authors' interpretation of rapamycin-treatment as inducer of autophagy. However, rapamycin, which inhibits mTOR has a range of cellular effects, most well described among those is the inhibition of mRNA translation induction. The authors' claim that autophagy is mediating the rapamycin effect is purely correlative but not supported by evidence. No causal or mechanistic link between autophagy and DNA repair is provided in the manuscript. The authors could claim that rapamycin treatment reduces gH2AX (which the authors confuse with DNA damage in their text as it is a marker that is associated with DNA damage sites). The link they observe between chromatin structure and RAD51 recruitment might be interesting, however, it remains unexplored. It was previously established that chromatin structure changes such as loss of cohesin and heterochromatin are associated with oocyte aging and genome instability, which might potentially be interesting to assess in the context of rapamycin treatment.

Specific comments:

1. The authors' claim that oocytes had limited DNA repair capacity is at odds with the very low mutation rates in oocytes. Almost all germline mutations are of paternal but not maternal origin and generally germline mutation rates are by more than an order of magnitude lower than somatic mutation rates.
2. Multiple controls such as rapamycin treatment without etoposide, IR etc. are lacking throughout.
3. In Figure 2K-M they use UV treatment and assess gH2AX. The authors need to follow here the UV-induced lesions, i.e. cyclobutane pyrimidine dimers (CPDs) for which specific antibodies are established (anti-CPD and anti-6-4PPs).
4. In Figure 3A-D RAD-51 doesn't colocalize with gH2AX. Why are no markers of NHEJ checked?
5. In Figure 3F-J the images are difficult to reconcile with the bar graph quantification shown. Clearly there is a lot of variability in the COMET assay that is not reflected in their quantification.
6. N as measure of independent biological repeats is missing in most figures, how many repetitions were performed?

Minor comments:

Suppl. 5C: p-values between cntrl. and Rapamycin treatment are not provided. Although rapamycin decreases DD it never returns to baseline.
Suppl. 5A-B: P-values not shown in between the groups

Reviewer #3

(Remarks to the Author)

This article focuses on the DNA damage in fully grown GV oocytes. It is a topic of interest to the researchers in the related

areas but the paper needs very significant improvement to improve the scientific premise of the paper. Most of the conclusions drawn in your manuscript are derived from immunofluorescence experiments and some are circumstantial rather than direct evidence, therefore do not meet the level of publication in Nature Communications journals. There are some suggestions for improvement:

1. In line 27 and figure 4F, the conclusion that oocyte failure to repair damaged DNA is due to the inability of DDR proteins to access altered "closed" chromatin conformation in DNA-damaged oocytes cannot be drawn by listing only one DDR protein, RAD51.
2. In line 279, the colocalization of RAD51 and DNA is verified by only one method. It can also be validated by co-immunoprecipitation (ChIP or CUT&Tag) combined with PCR.
3. As shown in the figure 2A-D, it indicates that there is a certain autophagy activity in oocytes, but the level of autophagy in oocytes after etoposide treatment decreases. Whether the DNA damage caused by this treatment is too severe, autophagy activity is reduced due to activation of the apoptosis pathway. Is it possible to detect apoptotic activity in oocytes after etoposide treatment?
4. The article writes that mouse and porcine oocytes fail to activate autophagy in response to DNA damage, is there no change of γ H2AX levels in aging oocytes or after etoposide-treated oocytes treated with spautin-1? In line 233, the result shown in the Supplementary Figure 2I-K seems contradictory to this view, suggests that DNA damage can induce autophagy in oocytes.
5. In line 390, the article writes that p53-mediated apoptosis is not functional in fully grown GV oocytes, but the cited literature identifies that p63 regulates the apoptosis pathway of GV oocytes, and the way described here is ambiguous, making people mistakenly believe that there is no apoptotic pathway in oocytes.
6. In line 289 and figure 4F, the relationship between RAD51 localization and chromatin conformational changes is correlated without direct evidence.
7. In line 294, you check chromatin status with alkaline comet assay (DNase sensitivity assay). Can you supplement the ATAC-seq experiment for verification?
8. The view that induction autophagy repair oocyte DNA damage is more one-sided for the improvement of overall oocyte quality, and excessive autophagy levels may reduce oocyte quality by affecting cytoplasmic maturation, when DNA repair is not meaningful, in other words, induction of autophagy may not be the best way to repair oocyte DNA damage.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

In response to my comments, and those of the other reviewers, the authors have provided additional controls and new experiments to support their existing data. I still find that the wording and conclusions drawn are too strong in context of the data presented. In particular, the main concept that aging-related aneuploidy is the result of reduced autophagy leading to a weakening of the DNA damage response and increased unrepaired DNA damage was not directly demonstrated.

Reviewer #2

(Remarks to the Author)

The authors have responded to some of the comments by additional experiments. The paper reports interesting results particularly regarding the effects of chromatin structure in the accessibility to double strand break repair factors in GV oocytes. However, the authors overstate their results in a misleading way ultimately compromising their credibility as pointed out before and again explained below.

For example, the authors indirectly induce DNA damage via the topoisomerase II inhibitor etoposide and indirectly assess DNA double strand break sites by γ H2AX and then generalize etoposide treatment and γ H2AX levels to all "DNA damage". This is misleading because of the wide variety of DNA damage types and repair mechanisms. In the case of oocytes the type of damage and outcome is of central importance because oocytes are the predominant source of aneuploidy but have only a very minor contribution to germline mutations. Therefore, precision of language when stating scientific results is an important part of scholarly publishing. Instead the authors directly insert their interpretations into the results.

The issue of their misleading overstatements is continued into the new results. As already pointed out before, rapamycin has multiple effects and how it relates to the observed induction of autophagy is unclear given the numerous other targets it has. The authors now use spautin-1, which has been linked to autophagy inhibition by inhibiting the ubiquitin-specific peptidase USP10. Instead of stating what spautin-1 really targets, the authors superficially (in a strict sense falsely) state "Spautin-1 is a specific and potent small molecule inhibitor that efficiently inhibits autophagy by degrading Beclin-1". As usual with chemical inhibitors, however, there is no unique specificity and instead it was recently published to also inhibit the mitochondrial complex I. To support their claims, the authors then use MG-132 and claim it as autophagy inhibitor, which again is at least a gross oversimplification because MG-132 is an inhibitor of the UPS proteasome, which while playing also a role in autophagy is a distinct part of the proteostasis network. Instead, the authors mislead again by stating "Proteasomal inhibitor, MG-132, is known to induce autophagy by increasing LC3-I to LC3-II conversion". While that may be a consequence of proteasome inhibition, there are multiple other consequences. Therefore, the authors' far reaching claim of a specific role of autophagy in the observed effects is insufficiently supported. The new results from the atg5 knockout mice they include in their response (but not in the manuscript) could shed light on this and could indeed overcome the limitations of the current study that relies exclusively on inhibitors (etoposide, rapamycin, spautin-1, MG132) all of which have the issue that their usage cannot support the claims on specificity that the authors put forward.

The misleading overstatements by the authors are found throughout the manuscript and compromise the scientific value of their work. Another typical example "...ATM kinase, the master regulator of DNA damage checkpoint... Therefore, phospho-ATM (p-ATM) is considered a sensitive marker of DNA damage sensing and repair." belongs in an opinion paper not in a research article as there are distinct DNA damage checkpoints some of which are regulated by ATM and ATM is involved e.g. in responding to DSBs but other damage types do not involve ATM.

Reviewer #3

(Remarks to the Author)

The authors have improved the manuscripts. However, similar to before, many conclusions still lack direct evidence, most conclusions drawn in your manuscript are derived from immunofluorescence experiments. For example, many published papers (PMID: 29720659; PMID: 28723896; PMID: 32169215) have showed the ATAC-seq to check chromatin status. The experiment is feasible in oocytes and very important to support the conclusion. However, the authors use DNase sensitivity assay and acH3K9/14 to check chromatin status. I think the results were indirect.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Thank you for including additional data in the manuscript. However, as raised in previous reviews, the wording of the manuscript and conclusions drawn are too strong throughout based on the data presented.

Reviewer #2

(Remarks to the Author)

The authors have adequately addressed my comments. They have now more precisely described their results and included data on the Atg5 knockout mice that strengthen their conclusions.

Reviewer #3

(Remarks to the Author)

I still think that the methods using DNase sensitivity assay and histone modification to check chromatin status were indirect. Many published papers (PMID: 29720659; PMID: 28723896; PMID: 32169215) have showed the ATAC-seq to check chromatin status in hundreds mouse oocytes and embryos.

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In this revised version, we have included new experiments. Summary of the major changes:

1- Reviewers 1 and 2: As suggested by Reviewer 1, to confirm that rapamycin's effect on chromatin structure, RAD51 localization at the DNA and DNA damage repair is specifically through autophagy and not through other mTOR-dependent effect, we included an additional group in all experiments, in which autophagy is inhibited in rapamycin-treated DNA-damaged oocytes using spautin-1 (a specific inhibitor for autophagy through beclin-1 degradation, an mTOR-independent mechanism). Importantly, we found that addition of spautin-1 abolished the effect of rapamycin on DNA damage repair, confirming that the observed effect of rapamycin on DNA damage repair is through autophagy induction (**Fig. 2E,F; Fig. 2G,H; Fig. 2I,J; Fig. 3A-C; Fig. 3D,E; Fig. 3F; Fig. 4A-C; Fig. 4D,E; Fig. 5A,B; Fig. 5C-E; Fig. 5F-H; Fig. 6A-C; Fig. 6D,E; Supplementary Fig. 1C,D; Supplementary Fig. 2F,G; Supplementary Fig. 2H-J; Supplementary Fig. 3A-C; Supplementary Fig. 4B,C; Supplementary Fig. 4E,F; Supplementary Fig. 4G,H; Supplementary Fig. 5A,B; Supplementary Fig. 6E,F; Supplementary Fig. 9C,D**).

2- Reviewers 1 and 2: We induced autophagy using another drug, MG-132 and assessed RAD51 localization in DNA-damaged oocytes. We found that inducing autophagy by MG-132 increased RAD51 localization at the DNA in etoposide-treated oocytes. To confirm that MG-132 effect on increasing RAD51 localization to the DNA is through autophagy induction, we have included an additional group, in which etoposide-treated oocytes were treated with both MG-132 and spautin-1. We found that addition of spautin-1 abolished the effect of MG-132 on RAD51 localization, further indicating that autophagy promotes DNA damage repair (**Supplementary Fig. 3**).

3- Reviewer 1: To understand how DNA damage leads to lagging chromosomes and aneuploidy, we included new data showing that chromosome fragmentation in DNA-damaged oocytes is caused by microtubule-induced tension on chromosomes and showed that almost all chromosome fragments in etoposide-treated oocytes are those containing kinetochores. The presence of separated kinetochores in DNA-damaged

oocytes might explain why homologous chromosomes do not properly segregate during anaphase/telophase I, leading to aneuploidy (**Supplementary Fig.1G,H**).

4- Reviewer 1: We have examined the effect of autophagy inhibition on chromatin structure (using 2 different methods, DNase sensitivity assay and acH3K9/14 staining), and found that autophagy inhibition by spautin-1 increased “closed” chromatin status, compared to control oocytes (**Fig. 4A-C and Fig. 4D,E**).

5- Reviewers 1, 2 and 3: We investigated the localization of Ku80, an identified NHEJ repair protein in full-grown oocytes, in response to DNA damage. Similar to RAD51, Ku80 localization to the DNA was not significantly increased following DNA damage, whereas inducing autophagy by rapamycin, but not by rapamycin+spautin-1, increased Ku80 localization to the DNA (**Supplementary Fig. 5A,B**).

6- Reviewers 1, 2 and 3: To confirm that “closed chromatin” structure is the cause, at least in part, of decreased RAD51 localization to the DNA in DNA-damaged oocytes, we increased “open chromatin” status using HDAC inhibitor, trichostatin A, in DNA-damaged oocytes and examined RAD51 localization. We found that increasing open chromatin status, by trichostatin A, increased RAD51 localization to the DNA in etoposide-treated oocytes (**Supplementary Fig. 7A,B**).

7- Reviewers 1, 2 and 3: To confirm that autophagy induction increases RAD51 localization to the DNA, at least in part, by promoting “open chromatin” structure, we investigated whether increasing closed chromatin state abolishes the increased RAD51 localization to the DNA in rapamycin-treated DNA-damaged oocytes. We found that treating the oocytes with garcinol, an HAT inhibitor that has been shown to increase “closed chromatin” structure, decreased RAD51 localization to the DNA in rapamycin-treated DNA-damaged oocytes (**Fig. 3A,B**).

8- Reviewers 1, 2 and 3: To confirm that autophagy increases RAD51 localization to the DNA, at least in part, by promoting “open chromatin” structure, we investigated whether increasing closed chromatin state (by PARP-1 inhibition) abolishes the increased RAD51 localization to the DNA in rapamycin-treated DNA-damaged oocytes. We found that inducing “closed chromatin” structure by inhibiting PARP1 abolished the “open chromatin status” in rapamycin-treated DNA-damaged oocytes (**Fig. 5C-E**), and, importantly, impaired RAD51 localization to the DNA (**Fig. 5F,H**).

9- Reviewer 2: We have repeated all experiments in which the effect of autophagy induction is examined to include rapamycin only group (**Fig. 2E,F; Fig. 2J,H; Fig. 2I,J; Fig. 3A-C; Fig. 3D,E; Fig. 3F; Fig. 4A-C; Fig. 4D,E; Fig. 5A,B; Fig. 5C-E; Fig. 5F-H; Fig. 6A,B; Fig. 6D,E; Supplementary Fig. 1C,D; Supplementary Fig. 2F,G; Supplementary Fig. 2H-J; Supplementary Fig. 3A-C; Supplementary Fig. 4B,C; Supplementary Fig. 4E,F; Supplementary Fig. 4G,H; Supplementary Fig. 5A,B; Supplementary Fig. 6E,F; Supplementary Fig. 9C,D**).

10- Reviewer 3: We have examined CPDs after UV radiation. We found that CPDs were significantly increased in UV-treated oocytes (**Supplementary Fig. 2F,G**).

11- Reviewer 3: To confirm the colocalization of RAD51 and DNA, we isolated DNA-protein complex using chromatin extraction protocol followed by western blot analysis of RAD51. Similar to our immunocytochemistry data, we found that RAD51 protein levels in the extracted chromatin did not vary significantly between control and etoposide-treated oocytes. Importantly, autophagy induction with rapamycin treatment, but not with rapamycin+spautin-1, significantly increased RAD51 levels in the extracted chromatin of etoposide-treated oocytes (**Fig. 3F**).

12- Reviewer 3: We have examined caspase 3 activity, a marker of apoptosis, and we did not find a significant difference between control and etoposide-treated oocytes (**Supplementary Figure 1B**).

13- Based on the new data we have rearranged the figures and currently the manuscript contains 7 main figures and 9 Supplementary figures.

Below are point-by-point responses.

Reviewer comments:

Reviewer #1:

We thank the reviewer very much for carefully reading our manuscript. We find the Reviewer's comments and suggestions to be extremely valuable for enhancing and strengthening our manuscript.

General comments:

1. In this study, the authors propose that oocyte failure to repair DNA damage is due to the inability of repair proteins to access closed chromatin and that this is due to lack of autophagy induction. I did not find that there was sufficient evidence to support this conclusion. Although there are some tantalising observations, these observations could be coincidental and the data as presented do not convincingly demonstrate that "DNA repair, chromatin configuration and autophagy are directly connected".

Thank you very much for raising this point. We have added new experiments and modified our language in the revised version to strengthen our conclusion and to address the reviewer's concern (i.e., demonstrating that DNA repair, chromatin configuration and autophagy are directly connected).

I- Demonstrating that **autophagy directly promotes DNA damage repair:**

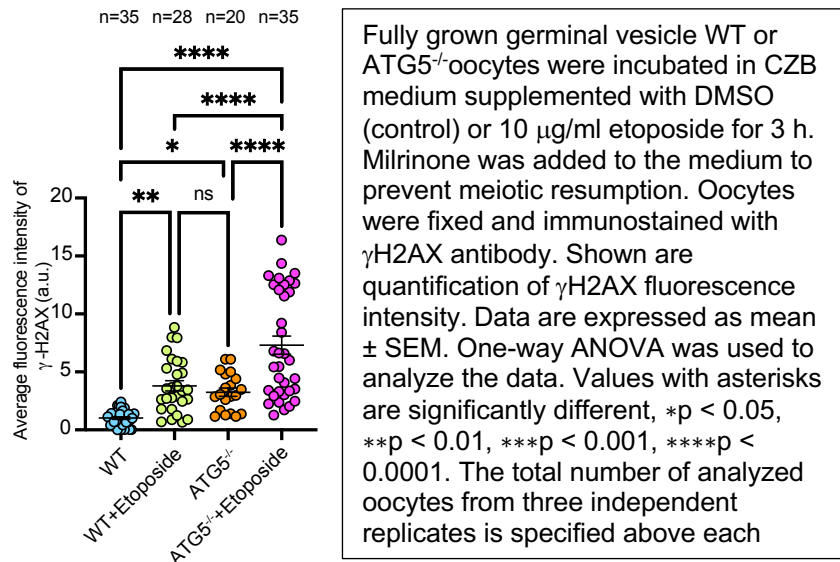
1. We found that autophagy inhibition by spautin-1 increased DNA breaks (hereafter referred to as DNA damage) as evidenced by increased γ -H2AX levels (**Fig. 2K,L**).

Moreover, inhibiting autophagy by spautin-1 further increased the level of DNA damage in etoposide-treated oocytes (**Fig. 2K,L**).

2. Our data revealed that autophagy induction by rapamycin enhanced DNA damage repair in etoposide-treated oocytes as evidenced by decreased γ -H2AX (**Fig. 2E,F**) and p-ATM (**Supplementary Fig. 4G,H**) levels and increased localization of RAD51 (**Fig. 3A,B**) and Ku80 (**Supplementary Fig. 5**) repair proteins to the DNA. To confirm that rapamycin's effect on DNA damage repair is specifically through autophagy and not through other mTOR-dependent effect, and as suggested by Reviewer 1 below, we included an additional group in all experiments (as suggested below), in which etoposide-treated oocytes were treated with both rapamycin and spautin-1 (a specific inhibitor for autophagy through beclin-1 degradation, an mTOR-independent mechanism). Importantly, we found that addition of spautin-1 abolished the improving effect of rapamycin on DNA damage repair (**Fig. 2E,F; Fig. 3A,B; Supplementary Fig. 4G,H and Supplementary Fig. 5**), indicating that rapamycin improves DNA damage repair by stimulating autophagy. Furthermore, the effect of autophagy induction on DNA damage repair, induced by etoposide, was confirmed using two additional DNA damage inducers, ultraviolet B radiation (UV) and ionizing radiation (IR) (**Fig. 2I,J; Fig. 3D,E and Supplementary Fig. 2H-J**).

3. To confirm our conclusion, we induced autophagy using another drug, MG-132. We found that inducing autophagy by MG-132 decreased γ -H2AX levels and increased RAD51 localization at the DNA in etoposide-treated oocytes. To confirm that MG-132 effect on reducing DNA damage level is through autophagy induction, we have included an additional group, in which etoposide-treated oocytes were treated with both MG-132 and spautin-1. We found that addition of spautin-1 abolished the improving effect of MG-132 on DNA damage, further indicating that autophagy promotes DNA damage repair (**Supplementary Fig. 3**).

Although not included in the manuscript, we examined an oocyte model in which autophagy is reduced. We generated mice deficient in Autophagy-Related Gene 5 (ATG5) specifically in oocytes (ATG5^{-/-} oocytes) by crossing ATG5 floxed allele mice with Gdf9-Cre mice. Our preliminary data show that ATG5^{-/-} oocytes exhibit higher levels of γ -H2AX when compared to WT oocytes. Moreover, ATG5^{-/-} oocytes were sensitive to a low level of DNA damage. Compared to WT oocytes, ATG5^{-/-} oocytes showed significantly higher levels of γ -H2AX following the exposure to a low concentration (10 μ g/ml) of etoposide treatment. However, because we are just starting our mouse colony, we are not able to get enough oocytes to carry out additional experiments and, therefore, these data were not included in the manuscript.



II- To demonstrate that **autophagy influences chromatin structure**:

1. We have examined the chromatin status using 2 different methods (DNase sensitivity assay and acH3K9/14 staining) and found that autophagy inhibition by spautin-1 increased “closed” chromatin status, compared to control oocytes. We have added this new data in **Fig. 4A-C and Fig. 4D,E**.

2. Using these 2 different methods to assess chromatin status (acH3K9/14 staining and DNase sensitivity assay), we found that autophagy stimulation by rapamycin increased “open chromatin” structure in DNA-damaged oocytes (induced by etoposide or IR). Again, to confirm that rapamycin effect on chromatin remodeling is through autophagy, we included an additional group, in which DNA-damaged oocytes (induced by etoposide or IR) were treated with both rapamycin and spautin-1. Importantly, we found that addition of spautin-1 abolished the effect of rapamycin on chromatin status, indicating that autophagy induction induces open chromatin status (**Fig. 4A-C; Fig. 4D,E and Supplementary Fig. 6**).

III- To demonstrate that **opened chromatin conformation caused by autophagy is required for efficient RAD51 localization** to damaged DNA sites.

In the revised version, we have included new experiments to manipulate the chromatin status using 3 different approaches (increasing open chromatin state by HDAC inhibition and increasing closed chromatin state by HAT or PARP-1 inhibition) to confirm our conclusion that, “opened chromatin conformation is required for efficient RAD51 localization to damaged DNA sites”:

Histone acetylation is a major regulator for chromatin status and largely regulated by histone deacetylases (HDACs) and histone acetyl transferases (HATs). Therefore, HAT inhibitors have been shown to induce “closed chromatin” status, whereas HDAC

inhibitors have been shown to induce “open chromatin” status (PMID: 28625481; PMID: 15292402; PMID: 31799598; PMID: 33875841).

1. Our data indicate that the chromatin is relatively closed in DNA damaged oocytes (**Fig. 4A-C; Fig. 4D,E and Supplementary Fig. 6**) with decreased RAD51 localization at the DNA (**Fig. 3**). To confirm that “closed chromatin” conformation is the cause, at least in part, of decreased RAD51 localization to the DNA in DNA-damaged oocytes, we increased “open chromatin” status using HDAC inhibitor, trichostatin A, in DNA-damaged oocytes and examined RAD51 localization. We found that increasing open chromatin status, by trichostatin A, increased RAD51 localization to the DNA in etoposide-treated oocytes, indicating that “closed chromatin” structure is the cause, at least in part, of decreased RAD51 localization to the DNA in DNA-damaged oocytes. We have added this new data in **Supplementary Fig. 7A,B**.

2. We found that, following DNA damage, the chromatin is relatively closed with reduced RAD51 localization to the DNA and autophagy induction (by rapamycin treatment but not by rapamycin+spautin-1) increased the level of open chromatin state and increased RAD51 localization to the DNA in DNA-damaged oocytes. Importantly, treating the oocytes with garcinol, an HAT inhibitor that has been shown to increase “closed chromatin” structure, decreased RAD51 localization to the DNA in rapamycin-treated DNA-damaged oocytes, indicating that autophagy increased RAD51 localization to the DNA, at least in part, by promoting “open chromatin” structure. We have added this new data in **Fig. 3A,B**.

3. PARP-1 regulates chromatin remodeling, and its activation promotes PARylation and “open chromatin” structure (PMID: 28676700; PMID: 34432161). We found that inducing more “closed chromatin” structure by inhibiting PARP1 abolished the “open chromatin status” in rapamycin-treated DNA-damaged oocytes (**Fig. 5C-E**) and importantly, impaired RAD51 localization to the DNA (new data, **Fig. 5F,H**), indicating that autophagy promotes RAD51 localization to the DNA, at least in part, by PARP-1-mediated “open chromatin” status.

Importantly, our findings are consistent with other publications (in non-oocyte models) that open chromatin status is required for DNA repair protein accessibility to damaged DNA (PMID: 24674626; PMID: 17893239; PMID: 11003660; PMID: 16936822; PMID: 25250043).

Taken together, we hope that the reviewer agrees with us that these results combined provide evidence that *DNA repair, chromatin configuration and autophagy are directly connected*.

2. It is stated in the abstract that DNA repair is not robust in mammalian oocytes. This claim is too broad as there is good evidence that immature oocytes have very robust DNA repair. The abstract should be specific about what stage oocytes are being referred to (i.e. fully grown). Furthermore, contrary to the major premise of this paper

that fully grown oocytes are deficient in DNA repair, there is evidence that fully-grown meiotically arrested GV oocytes can repair DNA damage (e.g. Collins 2015 and Cai 2023).

- Thank you very much for raising this important point. As suggested, we have specified the oocyte stage (full-grown oocytes) in the abstract.
- We agree with the reviewer's comment. We believe that the full-grown oocytes can "partially" repair DNA damage. However, as we mentioned in both the first and revised versions (**lines 185-186**) of the manuscript, we believe that full-grown GV oocytes have a limited capacity to repair DNA damage.
- Also, we believe that there is no contradiction in the provided papers with our conclusion. For example, in Cai 2023 (PMID: 3740758, Fig. 2B,C), the authors found that after 6 hour of etoposide washout, the level of DNA damage (γ -H2AX pixel intensity) is still comparable to that after 3 h of etoposide washout (albeit decreased from that at 0 h). Importantly, these levels of γ -H2AX pixel intensity (i.e. DNA damage levels after 3 or 6h post-etoposide washout) were at least 7-fold higher in 50 μ g/ml etoposide treatment and 12-fold higher in 100 μ g/ml etoposide treatment when compared to controls (non-etoposide-treated oocytes at 0h, Fig. 2B). These results are consistent with our conclusion that full-grown oocytes are not completely efficient in repairing DNA damage. Unfortunately, we can't compare our results to Collins 2015, because in that paper, the authors (1) used a low concentration of etoposide (25 μ g/ml) vs. 50 μ g/ml in our study, and (2) incubated the oocytes with etoposide for only 15 min vs. 3 h in our study. We already discussed this in our original submission (**lines 158-162**) and the new submission (**lines 181-186**) of the manuscript, which reads as "Given that oocytes can repair mild DNA damage (induced by a short incubation, 15 min, with a lower concentration, 25 μ g/ml, of etoposide) over a period of 10 h⁵², our findings suggest that full-grown GV oocytes have a limited capacity to repair moderate levels of DNA damage and, at that stage, one or more of DDR mechanisms are not fully functional". Furthermore, we have repeated our experiment with another hand, and we got similar results.

3. The introduction needs to more clearly explain in detail what is known about the role of autophagy, chromatin configuration and in the repair of DNA damage.

As suggested, we have included more detail in the introduction about the role of autophagy in chromatin configuration and DNA damage repair (**lines 88-93**).

4. As summarised in Figure 4 (schematic, top panel), and inferred by the focus of the introduction, the authors are proposing that age-associated aneuploidy is caused by DNA double strand breaks, leading to failure of chromosomes to segregate properly at meiosis 1. Conceptionally, it is not clear how DNA fragmentation caused by DNA dsDNA breaks would result in lagging chromosomes and chromosomal aneuploidies (like trisomy 21). In the schematic, they also show breaks in both chromatids of a

bivalent- was this intentional, as it implies the break occurred before DNA replication (which literature suggests would be efficiently repaired during prophase arrest, long before maternal aging occurs)?

- In the revised version (**Supplementary Fig.1G,H**), we investigated how DNA fragmentation caused by DNA breaks might result in lagging chromosomes and found that almost all chromosome fragments in etoposide-treated oocytes are those containing kinetochores. Therefore, we asked whether chromosome fragmentation at metaphase I stage in etoposide-treated oocytes is induced by microtubule-induced tension on chromosomes. Treatment of DNA-damaged oocytes with nocodazole, a microtubule depolymerizing drug decreased the chromosome fragmentation phenotype, indicating that microtubule-induced tension on chromosomes leads to chromosome fragmentation a metaphase I in DNA-damaged oocytes. During meiosis I, homologous chromosomes must be bioriented and correctly attached (at kinetochores) to opposite spindle poles, necessary for faithful chromosome segregation during anaphase I and telophase I. The presence of separated kinetochores in DNA-damaged oocytes can explain why homologous chromosomes could not properly segregate, leading to lagging chromosomes during anaphase/telophase I and aneuploidy at metaphase II. We have included this new data in **Supplementary Fig.1G,H and lines 152-166**.
- Thank you very much for raising this point, to which we completely agree. In the schematic diagram we did not intend to show breaks in both chromatids. To avoid this confusion, we have modified our schematic diagram to show breaks in only one chromatid (**Fig. 7**).

Specific comments

1. Line 98-99- it's not really unexpected that oocytes have differently to somatic cells. Oocytes do a lot of things differently to somatic cells. Suggest revising wording.

As suggested, we have modified the sentence and deleted the word “unexpectedly” from the abstract.

2. What is the direct evidence that it's the failure to activate autophagy that causes altered chromatin structure and failure of RAD51 to localise to sites of DNA damage, failed DNA repair and increased aneuploidy?

- Thank you very much for your comment. As explained in our response to **general comment #4** (above), we demonstrated that DNA damage leads to aneuploidy and provided an explanation of increased aneuploidy in DNA-damaged oocytes (**Supplementary Fig.1G,H and lines 152-166**).
- Moreover, as we described in our response to **general comment #1**(above), we demonstrated that autophagy directly promotes (1) chromatin remodeling, (2) RAD51 localization to the DNA and (3) DNA damage repair. Indeed, inducing autophagy in DNA-damaged oocytes increased open chromatin state, increased RAD51 localization to the DNA and decreased the level of DNA damage.

- In contrast to somatic granulosa cells (**Supplementary Fig.2B-E**) and mitotic cells (PMID: 27917193), we found that autophagy (as assessed by Cyto-ID and Western blot analysis) was not increased (even decreased) in DNA-damaged oocytes, indicating that full-grown oocytes fail to activate autophagy in response to DNA damage.
- Taken together and given that inducing autophagy in DNA-damaged oocytes increased open chromatin state, increased RAD51 localization to the DNA, decreased the level of DNA damage and decreased the incidence of aneuploidy, our data indicate that the failure to activate autophagy contributes to altered chromatin structure, the failure of RAD51 to localize to DNA-damaged sites, the failure of DNA repair and increased aneuploidy. However, we have tempered our language throughout the manuscript (e.g. **lines 449-451**).

3. Figure 1c- how were foci counted- H2AX is not distinct foci I these images?

Thank you very much for this valuable comment. What we presented is a z-project image (not a single slice image), in which several foci at different focal planes appear merged. The foci numbers were automatically calculated after 3D reconstruction (taking each single-slice image into consideration) using isosurface and spot analysis features of Bitplane Imaris software. Because we could not exclude the possibility that two closely associated foci could be counted as one, we employed the same parameters among all groups. Importantly, the quantification of γ -H2AX fluorescence intensity was consistent with the quantification of γ -H2AX foci numbers. However, we totally understand the reviewer's concern and, therefore, we removed all quantifications of γ -H2AX foci numbers and only presented the quantifications based on γ -H2AX fluorescence intensity, similar to several previous studies.

4. Figure 1 indicates that etoposide treated oocytes had twice the number of foci as controls, with a cluster of oocytes having the same number of foci as controls. Following on from this, it is stated that 40% of the oocytes progressed to MII- were these just the oocytes that had similar H2AX to controls?

Thank you very much for this observation. Although this is possible, unfortunately, we can't confirm this observation from the current data. After checking the raw data of **Fig. 1B**, ~20% of etoposide-treated oocytes have γ -H2AX levels comparable to (overlapping with those in) control oocytes. However, 40% of etoposide-treated oocytes extruded the polar body (**Supplementary Fig. 1C**), indicating that ~50% of etoposide-treated oocytes with a relatively high DNA damage (compared to controls) were able to extrude the polar body. However, we agree with the reviewer's comment and can't exclude that those etoposide-treated arrested oocytes might have a very high levels of DNA damage. We have added this possibility to the text (**lines 471-472**) to read, "Indeed similar to a previous report⁵¹, we found that ~40% of etoposide-treated oocytes that underwent NEBD were able to extrude the polar body, likely because they have DNA damage below the threshold of SAC activation".

5. The legend for Figure 1 E-K indicates that the oocytes were released from arrest and then allowed to progress through to MII with no period of recovery. It seems very unlikely that chromosomes could segregate properly if there are up to 150 DSBs that have not been given time to repair- and yet only a single lagging chromosome is seen, rather than DNA fragments scattered all over the place, as reported by others. How can this be explained?

Thank you very much for raising this point. We would like to clarify that the oocytes were exposed to DNA damage at GV stage (0 h) and examined for lagging chromosomes at anaphase I/telophase I stage which is ~9 h after the release from the arrest. So, we think that there is a considerable time for a relative recovery without etoposide (~ 8-9 h) before the chromosomes attempt to segregate. However, we do not think that there is a single lagging chromosome in the provided image. In fact, in the provided movie (**Supplementary Movie 2**) and Fig. 1E (currently **Fig. 1G**; the high-quality version), it is clear that ~50% of the chromosomes are lagged, but they are aggregated together and might appear as a few lagging chromosomes. It is of note, that following telophase I, metaphase II chromosome fragments can be scattered all over the place.

6. Figure 1 I-K. It is not clear what is meant by “compact” chromosome morphology? I agree the DNA looks different after DNA damage, but not compact. Indeed, the DNA looks more diffuse in the treated oocytes. The phenotypes of UV and IR exposed DNA do not look the same as etoposide, and I’m not really sure what the authors are getting at here because of course the morphology of damaged and fragmented DNA is different to intact DNA.

In Fig. 1I-K (currently **Fig. 1K-M**), we characterize the phenotype of chromosomes in DNA-damaged oocytes. We apologize if our term “compact” was a bit vague. We have now used the term “abnormal chromosome condensation” instead of “compact” and modified our text to read, “In contrast to control oocytes in which chromosomes have normal bivalent morphology, etoposide-treated oocytes had significantly higher rates of abnormal chromosome condensation (i.e., the loss of normal bivalent morphology) (**lines 169-170**). This loss of normal bivalent morphology of chromosomes (chromosome condensation defects) is consistent among etoposide-, UV- and IR-treated oocytes (**Fig. 1K-M**).

7. Fig 1 I-K. Did the authors really expose the oocytes to 100Gy IR? This is an extraordinarily high dose.

Thank you very much for this important observation. We used 100cGy which is 1Gy. We have corrected this “typo” mistake.

8. Fig 1 L-N- this is contrary to other reports showing that fully grown GV's can repair high levels of etoposide induced DNA damage (e.g. Collins 2015, Cai et al 2023).

Thank you very much for raising this important point. Please find our response to general comment #2 (above).

9. Fig 1G and H are not referred to in the text.

We apologize if this was not clear in the first version. Fig. 1G and H (currently **Fig. 1I,J**) were referred in the text of the original submission in line 137 (**line 146** in this revised version).

10. 40% of oocytes progress to MII, how many to MI?

We apologize if our writing was not clear. The % of MII oocytes were calculated from the oocytes underwent NEBD. We have clarified this in the revised manuscript (**line 136**).

11. Rapamycin is an inhibitor of the mTOR pathway, which has diverse roles. It is not specific to autophagy induction. The rationale for using rapamycin to rescue DNA damage is not clearly explained in the text. The authors frequently refer to autophagy induction by rapamycin leading to a certain result or outcome, but it's not possible to say if the autophagy or some other aspect of mTOR inhibition. E.g. it is concluded that "inefficient autophagy activation contributes to closed chromatin configuration in DNA damage oocytes" line 315-316. But only a handful of experiments (Figure 2E-G) in this paper were conducted with autophagy inhibitor (spautin-1), thus many of the observed rapamycin effects cannot be directly linked to autophagy at this stage.

Thank you very much for this valuable comment to which we completely agree. That is why, in Fig. 2E (original submission), we included an additional group in which etoposide-treated oocytes were treated with both rapamycin and spautin-1 (a specific inhibitor for autophagy through beclin-1, an mTOR-independent mechanism) to confirm that rapamycin effect on DNA damage repair is through autophagy and not through another mTOR-dependent effect. Because spautin-1 abolished the improving effect of rapamycin on DNA damage and because we got similar results using another autophagy inducer, MG-132, this initially led us to omit rapamycin+spautin-1 group in the other experiments. However, we totally understand the reviewer's concern and, as requested, we have repeated almost all experiments, in which DNA-damaged oocytes were treated with "rapamycin" to include an additional "Rapamycin+Spautin-1" group (**Fig. 2E,F; Fig. 2G,H; Fig. 2I,J; Fig. 3A-C; Fig. 3D,E; Fig. 3F; Fig. 4A-C; Fig. 4D,E; Fig. 5A,B; Fig. 5C-E; Fig. 5F-H; Fig. 6A-C; Fig. 6D,E; Supplementary Fig. 1C,D;**

Supplementary Fig. 2F,G; Supplementary Fig. 2H-J; Supplementary Fig. 3A-C; Supplementary Fig. 4B,C; Supplementary Fig. 4E,F; Supplementary Fig. 4G,H; Supplementary Fig. 5A,B; Supplementary Fig. 6E,F; Supplementary Fig. 9C,D). These experiments confirmed that the effect of rapamycin on DNA damage repair is through autophagy activation because spautin-1 abolished the effect of rapamycin on DNA damage repair. Moreover, we confirmed the role of autophagy in DNA damage repair using MG-132 to activate autophagy through an mTOR-independent pathway and showed that MG-132 decreased DNA damage in etoposide-treated oocytes and such DNA damage repair was abolished when spautin-1 was added to MG-132+etoposide group (**Supplementary Fig. 3A-C**).

12. The loss and induction of LC3II does not look very convincing in Figure 2E. How many oocytes and how many replicates?

There is no “LC3II” in Fig. 2E. If the question regarding Fig. 2C, we used 150 oocytes in each group, and the experiment was repeated 3 times. We apologize if the western blot image was not clear. We have replaced the representative western blot figure (**Fig. 2C**) and confirmed our western blot results by using an autophagy detection kit (**Fig. 2A,B**).

13. Figure 2 A/B- Why is the autophagy activity reduced after DNA damage?

Thank you very much for this important observation. As suggested by reviewer 3 (below) and based on our data, we believe that autophagy plays a role in DNA damage repair and that fully grown oocytes contain certain activity of autophagy (but not devoid of autophagy, as shown in control oocytes in **Fig. 2A,C**). This basal level of autophagy is likely protecting the oocyte against the mild threatening by endogenous DNA assaults. So, upon the exposure to moderate DNA damage (as in our study), it is expected that this basal level of autophagy available in the oocyte is consumed in response to initial DNA damage. However, the oocyte fails to activate additional autophagy, leading to reduced autophagy after DNA damage. This is supported by our data where autophagy inhibition by spautin-1 increased the level of DNA damage and further increased the level of DNA damage in etoposide-treated oocytes (**Fig. 2K-L and Fig. 2G,H**). Together, our data suggest a model that the full-grown oocyte contains a certain basal level of autophagy which is consumed in the initial response to DNA damage. However, due to the failure to further activate autophagy, the oocyte has a limited capacity to repair moderate/severe DNA damage. This model is supported by our data that autophagy induction promotes DNA damage repair following DNA damage. We have added this model to the text in **lines 485-488**.

14. Line 198- weakened DDR as compared to what?

We apologize if our term was vague. As suggested, we have replaced the word “weakened” by “deficient” in **line 220**.

15. Figure 2B is missing an etop + rapamycin group.

The purpose of this experiment was just to show that autophagy is not increased after DNA damage. However, as requested, we have repeated the experiment and added “etop + rapamycin” group to **Fig. 2A,B**.

16. Ref 36 is a conference abstract.

As suggested, we have deleted Ref 36.

17. What is Nir in Figure 3G and H? This is not explained in the figure legend.

We are sorry if this abbreviation was vague. We have mentioned the full name in the figure legend “Niraparib”, PARP-1 inhibitor (currently **Fig. 5**).

18. Line 254- It was stated that inducing autophagy (exposing to rapamycin) before inducing DNA damage reduces H2AX foci, and it was then concluded that autophagy reduces DNA damage. How do the authors know that rapamycin didn't interfere with the phosphorylation of histone H3 rather than actually reducing DNA breaks? Were comet assays performed to confirm this? Additionally, Spautin-1 should be used to confirm that this phenomenon is autophagy related. Similarly, although rapamycin reduced pATM levels, this could be unrelated to autophagy.

Thank you very much for this valuable comment. We did not conduct comet assays in this experiment. However, as requested, we have repeated this experiment (**Supplementary Fig. 4B,C**) with adding “spautin-1” to “etop + Rap” group and confirmed that this phenomenon is autophagy related and it is not caused by the interference of rapamycin with histone phosphorylation because the phosphorylation of histone increased after spautin-1 supplementation in the presence of rapamycin. Moreover, we have repeated the p-ATM experiment, added “spautin-1” to “etop + Rap” group and confirmed that this p-ATM reduction is due to autophagy activation (**Supplementary Fig. 4G,H**).

19. Line 275-282 (Figure 3). Elevated levels of RAD51 were not present after exposure to etoposide or UV. This is probably because there is a switch from dependence on HR to NHEJ in fully grown oocytes. The authors should also use a marker of NHEJ repair. Interestingly Rapamycin did increase RAD51 foci (rescue is not the right term in this instance), possibly due to impairment of the switch to NHEJ (notably, in Rapamycin can actually suppress both HR and NHEJ). Whether this is related to autophagy in oocytes is not known and cannot be determined from the data as presented.

Thank you very much for raising this interesting point. As requested, we have investigated Ku80, a recently identified NHEJ repair protein in full-grown oocytes (PMID: 37407587). Similar to RAD51, Ku80 localization to the DNA was not significantly increased following DNA damage, whereas inducing autophagy by rapamycin, but not by rapamycin+spautin-1, increased Ku80 localization to the DNA. We have added these new data in **Supplementary Fig. 5A,B**. These results further support our conclusion that autophagy promotes the localization of the DNA repair proteins, at least RAD51 and Ku80 (markers of HR and NHEJ, respectively), to the damaged DNA.

20. Experiments were done to demonstrate that oocytes might have a more closed chromatin structure after damage (and this this structure could be opened with Rapamycin). But whether this closed structure means that DNA repair proteins can not access DNA breaks is not clear.

In the revised version, we have included new experiments to manipulate the chromatin status using 3 different approaches (increasing open chromatin state by HDAC inhibition and increasing closed chromatin state by HAT or PARP-1 inhibition) to confirm our conclusion that, “opened chromatin conformation is required for efficient RAD51 localization to damaged DNA sites”:

Histone acetylation is a major regulator for chromatin status and largely regulated by histone deacetylases (HDACs) and histone acetyl transferases (HATs). Therefore, HAT inhibitors have been shown to induce “closed chromatin” status, whereas HDAC inhibitors have been shown to induce “open chromatin” status (PMID: 28625481; PMID: 15292402; PMID: 31799598; PMID: 33875841).

1. Our data indicate that the chromatin is relatively closed in DNA damaged oocytes (**Fig. 4A-C; Fig. 4D,E and Supplementary Fig. 6**) with decreased RAD51 localization at the DNA (**Fig. 3**). To confirm that “closed chromatin” conformation is the cause, at least in part, of decreased RAD51 localization to the DNA in DNA-damaged oocytes, we increased “open chromatin” status using HDAC inhibitor, trichostatin A, in DNA-damaged oocytes and examined RAD51 localization. We found that increasing open chromatin status, by trichostatin A, increased RAD51 localization to the DNA in etoposide-treated oocytes, indicating that “closed chromatin” structure is the cause, at least in part, of decreased RAD51 localization to the DNA in DNA-damaged oocytes. We have added this new data in **Supplementary Fig. 7A,B**.

2. We found that, following DNA damage, the chromatin is relatively closed with reduced RAD51 localization to the DNA and autophagy induction (by rapamycin treatment but not by rapamycin+spautin-1) increased the level of open chromatin state and increased RAD51 localization to the DNA in DNA-damaged oocytes. Importantly, treating the oocytes with garcinol, an HAT inhibitor that has been shown to increase “closed chromatin” structure, decreased RAD51 localization to the DNA in rapamycin-treated DNA-damaged oocytes, indicating that autophagy increased RAD51 localization

to the DNA, at least in part, by promoting “open chromatin” structure. We have added this new data in **Fig. 3A,B**.

3. PARP-1 regulates chromatin remodeling, and its activation promotes PARylation and “open chromatin” structure (PMID: 28676700; PMID: 34432161). We found that inducing more “closed chromatin” structure by inhibiting PARP1 abolished the “open chromatin status” in rapamycin-treated DNA-damaged oocytes (**Fig. 5C-E**) and importantly, impaired RAD51 localization to the DNA (new data, **Fig. 5F,H**), indicating that autophagy promotes RAD51 localization to the DNA, at least in part, by PARP-1-mediated “open chromatin” status.

Importantly, our findings are consistent with previous publications (in non-oocyte models) that open chromatin status is required for DNA repair protein accessibility to damaged DNA (PMID: 24674626; PMID: 17893239; PMID: 11003660; PMID: 16936822; PMID: 25250043).

Reviewer #2:

In their manuscript “Diminished autophagy activation is a crucial factor for weakened DNA damage repair in full-grown oocytes” Sun et al. claim that aneuploidy in oocytes is linked to reduced autophagy induction following DNA damage. Upon etoposide treatment, they observe gH2AX induction that they could reduce by rapamycin treatment. The central argument of the paper is the authors’ interpretation of rapamycin-treatment as inducer of autophagy. However, rapamycin, which inhibits mTOR has a range of cellular effects, most well described among those is the inhibition of mRNA translation induction. The authors’ claim that autophagy is mediating the rapamycin effect is purely correlative but not supported by evidence. No causal or mechanistic link between autophagy and DNA repair is provided in the manuscript. The authors could claim that rapamycin treatment reduces gH2AX (which the authors confuse with DNA damage in their text as it is a marker that is associated with DNA damage sites). The link they observe between chromatin structure and RAD51 recruitment might be interesting, however, it remains unexplored. It was previously established that chromatin structure changes such as loss of cohesin and heterochromatin are associated with oocyte aging and genome instability, which might potentially be interesting to assess in the context of rapamycin treatment.

We thank the reviewer very much for carefully reviewing the manuscript, and for the constructive feedback that have significantly improved and strengthen the manuscript.

1- The authors’ claim that autophagy is mediating the rapamycin effect is purely correlative but not supported by evidence.

Thank you very much for this valuable comment to which we completely agree. As suggested by reviewer 1, to confirm that the effect of rapamycin on DNA-damaged oocytes is through autophagy induction, we have included an additional group in all experiments, in which spautin-1 (a specific inhibitor for autophagy through beclin-1 degradation, an mTOR-independent mechanism) is added to rapamycin-treated oocytes. These experiments confirmed that the effect of rapamycin on DNA damage repair is through autophagy activation because spautin-1 abolished the effect of rapamycin on DNA damage repair (**Fig. 2E,F; Fig. 2G,H; Fig. 2I,J; Fig. 3A-C; Fig. 3D,E; Fig. 3F; Fig. 4A-C; Fig. 4D,E; Fig. 5A,B; Fig. 5C-E; Fig. 5F-H; Fig. 6A-C; Fig. 6D,E; Supplementary Fig. 1C,D; Supplementary Fig. 2F,G; Supplementary Fig. 2H-J; Supplementary Fig. 3A-C; Supplementary Fig. 4B,C; Supplementary Fig. 4E,F; Supplementary Fig. 4G,H; Supplementary Fig. 5A,B; Supplementary Fig. 6E,F; Supplementary Fig. 9C,D**). Moreover, we confirmed the role of autophagy in DNA damage repair using MG-132 to activate autophagy through an mTOR-independent pathway and showed that MG-132 decreased DNA damage in etoposide-treated oocytes and such DNA damage repair was abolished when spautin-1 was added to MG-132+etoposide group (**Supplementary Fig. 3A-C**). Furthermore, the role of autophagy in DNA damage repair is confirmed through autophagy inhibition. We found that autophagy inhibition by spautin-1 increased DNA breaks (hereafter referred to as

DNA damage) as evidenced by increased γ -H2AX levels (**Fig. 2K,L**). Moreover, inhibiting autophagy by spautin-1 further increased the level of DNA damage in etoposide-treated oocytes (**Fig. 2K,L**).

2- No causal or mechanistic link between autophagy and DNA repair is provided in the manuscript. The authors could claim that rapamycin treatment reduces γ H2AX (which the authors confuse with DNA damage in their text as it is a marker that is associated with DNA damage sites). The link they observe between chromatin structure and RAD51 recruitment might be interesting, however, it remains unexplored.

Thank you very much for raising these important comments.

We apologize if the term “DNA damage” is confusing. γ H2AX is recognized as a sensitive molecular marker for DNA damage breaks. However, for simplicity and to be consistent with other literature in the field, we mentioned in **line 120** “DNA breaks (hereafter referred to as DNA damage)”.

As mentioned in our response to Reviewer 1, we modified our experimental design and conducted additional experiments, in the revised version, to provide causal and mechanistic link between autophagy and DNA repair and to show that open chromatin structure caused by autophagy is required for efficient RAD51 localization to DNA damage sites.

I- Demonstrating that **autophagy directly promotes DNA damage repair**:

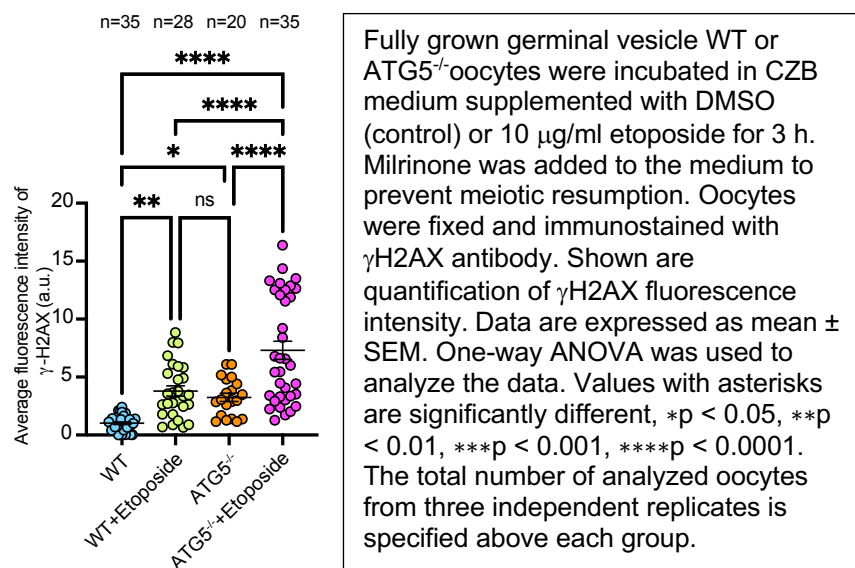
1. We found that autophagy inhibition by spautin-1 increased DNA breaks (hereafter referred to as DNA damage) as evidenced by increased γ -H2AX levels (**Fig. 2K,L**). Moreover, inhibiting autophagy by spautin-1 further increased the level of DNA damage in etoposide-treated oocytes (**Fig. 2K,L**).

2. Our data revealed that autophagy induction by rapamycin enhanced DNA damage repair in etoposide-treated oocytes as evidenced by decreased γ -H2AX (**Fig. 2E,F**) and p-ATM (**Supplementary Fig. 4G,H**) levels and increased localization of RAD51 (**Fig. 3A,B**) and Ku80 (**Supplementary Fig. 5**) repair proteins to the DNA. To confirm that rapamycin's effect on DNA damage repair is specifically through autophagy and not through other mTOR-dependent effect, and as suggested by Reviewer 1, we included an additional group in all experiments (as suggested below), in which etoposide-treated oocytes were treated with both rapamycin and spautin-1 (a specific inhibitor for autophagy through beclin-1 degradation, an mTOR-independent mechanism). Importantly, we found that addition of spautin-1 abolished the improving effect of rapamycin on DNA damage repair (**Fig. 2E,F; Fig. 3A,B; Supplementary Fig. 4G,H and Supplementary Fig. 5**), indicating that rapamycin improves DNA damage repair by stimulating autophagy. Furthermore, the effect of autophagy induction on DNA damage repair, induced by etoposide, was confirmed using two additional DNA damage

inducers, ultraviolet B radiation (UV) and ionizing radiation (IR) (**Fig. 2I,J; Fig. 3D,E and Supplementary Fig. 2H-J**).

3. To confirm our conclusion, we induced autophagy using another drug, MG-132. We found that inducing autophagy by MG-132 decreased γ -H2AX levels and increased RAD51 localization at the DNA in etoposide-treated oocytes. To confirm that MG-132 effect on reducing DNA damage level is through autophagy induction, we have included an additional group, in which etoposide-treated oocytes were treated with both MG-132 and spautin-1. We found that addition of spautin-1 abolished the improving effect of MG-132 on DNA damage, further indicating that autophagy promotes DNA damage repair (New **Supplementary Fig. 3**).

Although not included in the manuscript, we examined an oocyte model in which autophagy is reduced. We generated mice deficient in Autophagy-Related Gene 5 (ATG5) specifically in oocytes (ATG5^{-/-} oocytes) by crossing ATG5 floxed allele mice with Gdf9-Cre mice. Our preliminary data show that ATG5^{-/-} oocytes exhibit higher levels of γ -H2AX when compared to WT oocytes. Moreover, ATG5^{-/-} oocytes were sensitive to a low level of DNA damage. Compared to WT oocytes, ATG5^{-/-} oocytes showed significantly higher levels of γ -H2AX following the exposure to a low concentration (10 μ g/ml) of etoposide treatment. However, because we are just starting our mouse colony, we are not able to get enough oocytes to carry out additional experiments and, therefore, these data were not included in the manuscript.



II- To demonstrate that **autophagy influences chromatin structure**:

1. We have examined the chromatin status using 2 different methods (DNase sensitivity assay and acH3K9/14 staining) and found that autophagy inhibition by spautin-1 increased “closed” chromatin status, compared to control oocytes. We have added this new data in **Fig. 4A-C and Fig. 4D,E**.

2. Using these 2 different methods to assess chromatin status (acH3K9/14 staining and DNase sensitivity assay), we found that autophagy stimulation by rapamycin increased “open chromatin” structure in DNA-damaged oocytes (induced by etoposide or IR). Again, to confirm that rapamycin effect on chromatin remodeling is through autophagy, we included an additional group, in which DNA-damaged oocytes (induced by etoposide or IR) were treated with both rapamycin and spautin-1. Importantly, we found that addition of spautin-1 abolished the effect of rapamycin on chromatin status, indicating that autophagy induction induces open chromatin status (**Fig. 4A-C; Fig. 4D,E and Supplementary Fig. 6**).

III- To demonstrate that **opened chromatin conformation caused by autophagy is required for efficient RAD51 localization** to damaged DNA sites.

In the revised version, we have included new experiments to manipulate the chromatin status using 3 different approaches (increasing open chromatin state by HDAC inhibition and increasing closed chromatin state by HAT or PARP-1 inhibition) to confirm our conclusion that, “opened chromatin conformation is required for efficient RAD51 localization to damaged DNA sites”:

Histone acetylation is a major regulator for chromatin status and largely regulated by histone deacetylases (HDACs) and histone acetyl transferases (HATs). Therefore, HAT inhibitors have been shown to induce “closed chromatin” status, whereas HDAC inhibitors have been shown to induce “open chromatin” status (PMID: 28625481; PMID: 15292402; PMID: 31799598; PMID: 33875841).

1. Our data indicate that the chromatin is relatively closed in DNA damaged oocytes (**Fig. 4A-C; Fig. 4D,E and Supplementary Fig. 6**) with decreased RAD51 localization at the DNA (**Fig. 3**). To confirm that “closed chromatin” conformation is the cause, at least in part, of decreased RAD51 localization to the DNA in DNA-damaged oocytes, we increased “open chromatin” status using HDAC inhibitor, trichostatin A, in DNA-damaged oocytes and examined RAD51 localization. We found that increasing open chromatin status, by trichostatin A, increased RAD51 localization to the DNA in etoposide-treated oocytes, indicating that “closed chromatin” structure is the cause, at least in part, of decreased RAD51 localization to the DNA in DNA-damaged oocytes. We have added this new data in **Supplementary Fig. 7A,B**.

2. We found that, following DNA damage, the chromatin is relatively closed with reduced RAD51 localization to the DNA and autophagy induction (by rapamycin treatment but not by rapamycin+spautin-1) increased the level of open chromatin state and increased RAD51 localization to the DNA in DNA-damaged oocytes. Importantly, treating the oocytes with garcinol, an HAT inhibitor that has been shown to increase “closed chromatin” structure, decreased RAD51 localization to the DNA in rapamycin-treated DNA-damaged oocytes, indicating that autophagy increased RAD51 localization to the DNA, at least in part, by promoting “open chromatin” structure. We have added this new data in **Fig. 3A,B**.

3. PARP-1 regulates chromatin remodeling, and its activation promotes PARylation and “open chromatin” structure (PMID: 28676700; PMID: 34432161). We found that inducing more “closed chromatin” structure by inhibiting PARP1 abolished the “open chromatin status” in rapamycin-treated DNA-damaged oocytes (**Fig. 5C-E**) and importantly, impaired RAD51 localization to the DNA (new data, **Fig. 5F,H**), indicating that autophagy promotes RAD51 localization to the DNA, at least in part, by PARP-1-mediated “open chromatin” status.

Importantly, our findings are consistent with previous publications (in non-oocyte models) that open chromatin status is required for DNA repair protein accessibility to damaged DNA (PMID: 24674626; PMID: 17893239; PMID: 11003660; PMID: 16936822; PMID: 25250043).

Taken together, we hope that the reviewer agrees with us that these results combined provide evidence that autophagy directly regulates DNA damage repair by promoting open chromatin structure, a necessary step for the recruitment of DNA damage repair proteins to the DNA damage sites.

3- It was previously established that chromatin structure changes such as loss of cohesin and heterochromatin are associated with oocyte aging and genome instability, which might potentially be interesting to assess in the context of rapamycin treatment.

We thank the reviewer for this valuable suggestion. We totally agree that it is interesting to assess the effect of rapamycin on cohesin and chromatin structure changes. Aging-associated chromatin structure changes are more complex than previously believed. Age-associated chromatin structure changes are not universal, but it is cell-type specific. For example, in aged mice, a decrease of heterochromatin was found in excitatory neurons, but not in inhibitory neurons, heart, bone marrow or skeletal muscles (PMID: 36207411). Even there is a decline in heterochromatin at several locations, aging also correlates with an increased formation of localized heterochromatin at specific genomic loci (heterochromatin redistribution phenomenon, PMID: 18423606; PMID: 22647267). Because DNA-damaged sites are very random and unpredictable and because there are technical limitations regarding the use of mammalian oocytes (discussed below), it is technically infeasible to examine chromatin structural changes specifically at age-associated DNA-damaged sites in oocytes. Moreover, in full-grown oocytes, inducing DNA damage by etoposide does not affect cohesin as assessed by Rec8 localization (PMID: 16855401) and the loss of cohesin happens slowly during the aging process. Given that the focus of this study is to determine the response of full-grown oocytes to DNA damage repair mechanism (not specifically rapamycin), we feel that assessing the effect of rapamycin on oocyte cohesin and chromatin structure changes (after developing/optimizing the necessary techniques) are beyond the scope of this manuscript. However, the Reviewer's suggestions open interesting directions for future research, and therefore, assessing the effect of rapamycin on cohesin and age-associated genome instability, spanning different stages of oocyte aging, will be one of our future interests.

Specific comments:

1. The authors' claim that oocytes had limited DNA repair capacity is at odds with the very low mutation rates in oocytes. Almost all germline mutations are of paternal but not maternal origin and generally germline mutation rates are by more than an order of magnitude lower than somatic mutation rates.

We agree that the mutation rates are higher in male germline compared to female germline. However, the cause behind this observation is still controversial and one potential explanation is that around the time of birth, oocytes are arrested at the dictyate stage of meiotic prophase I with no subsequent DNA replication, whereas sperm cells divide continuously throughout the male reproductive life.

We want to clarify that we do not claim that “all” stages of oocytes had limited DNA repair capacity. Instead, this study focuses only on fully grown prophase I oocytes. Indeed, it is already acknowledged in the field that nongrowing and growing oocytes (but not full-grown oocytes) have a robust DNA damage response and as mentioned in **lines 500-503** “Similar to somatic cells, quiescent (nongrowing) and growing prophase I oocytes (from prepubertal mice) can efficiently repair severe DNA damage in apoptosis-inhibited mice by activating ATM, phosphorylating histone H2AX, and translocated RAD51 to the DNA-damaged sites”. Our data and others indicate that full-grown oocytes (the pool of oocytes which will be recruited for maturation) have a limited capacity to repair DNA damage.

2. Multiple controls such as rapamycin treatment without etoposide, IR etc. are lacking throughout.

Thank you very much for this valuable comment. We apologize for not including rapamycin only group in all experiments in the first version of the manuscript. Our reasons were because (1) we examined the effect of rapamycin alone in Fig. 2E-F (original submission), (2) the purpose of the remaining experiments was to examine the effect of rapamycin treatment in the presence of DNA damage. So, rapamycin+etoposide group is compared to etoposide only group (our proper control in this case) and (3) to minimize the use of mice according to the ethical framework for improving laboratory animal welfare. However, we understand the reviewer's concern and, as requested, we have repeated all experiments in which the effect of autophagy induction is examined, along with new experiments, and included rapamycin only group (**Fig. 2E,F; Fig. 2J,H; Fig. 2I,J; Fig. 3A-C; Fig. 3D,E; Fig. 3F; Fig. 4A-C; Fig. 4D,E; Fig. 5A,B; Fig. 5C-E; Fig. 5F-H; Fig. 6A,B; Fig. 6D,E; Supplementary Fig. 1C,D; Supplementary Fig. 2F,G; Supplementary Fig. 2H-J; Supplementary Fig. 3A-C; Supplementary Fig. 4B,C; Supplementary Fig. 4E,F; Supplementary Fig. 4G,H; Supplementary Fig. 5A,B; Supplementary Fig. 6E,F; Supplementary Fig. 9C,D**).

3. In Figure 2K-M they use UV treatment and assess gH2AX. The authors need to

follow here the UV-induced lesions, i.e. 22yclobutene pyrimidine dimers (CPDs) for which specific antibodies are established (anti-CPD and anti-6-4PPs).

Thank you very much for this valuable comment. As requested, we have examined CPDs after UV radiation. As shown in **Supplementary Fig. 2F,G**, CPDs were significantly increased in UV-treated oocytes.

4. In Figure 3A-D RAD-51 doesn't colocalize with γ H2AX. Why are no markers of NHEJ checked?

RAD51 localization to DNA-damaged sites (labeled by γ -H2AX) is essential for DNA damage repair. Following the repair, γ -H2AX signals significantly decrease (below the imaging threshold) before the complete release of RAD51. Therefore, it is possible to see some RAD51 foci at the DNA that do not colocalize with γ -H2AX. As suggested, we have investigated Ku80, a recently identified NHEJ repair protein in full-grown oocytes (PMID: 37407587). Similar to RAD51, Ku80 localization to the DNA was not significantly increased following DNA damage, whereas inducing autophagy by rapamycin, but not by rapamycin+spautin-1, increased Ku80 localization to the DNA. We have added these new data in **Supplementary Fig. 5A,B**. These results further support our conclusion that autophagy promotes the localization of the DNA repair proteins, at least RAD51 and Ku80 (markers for HR and NHEJ, respectively), to the damaged DNA.

5. In Figure 3F-J the images are difficult to reconcile with the bar graph quantification shown. Clearly there is a lot of variability in the COMET assay that is not reflected in their quantification.

The quantifications represent the ratio of comet length and moment between DNase-treated and non-DNase-treated values. We have repeated this experiment after including rapamycin only group and etoposide+rapamycin+spautin-1 group. We have replaced the "bar graph" quantifications with "scattered plot" (**Fig. 5D,E** in the new submission).

6. N as measure of independent biological repeats is missing in most figures, how many repetitions were performed?

As suggested, we have added the number of replicates in the corresponding figure legends of all figures.

Minor comments:

Suppl. 5C: p-values between cntrl. and Rapamycin treatment are not provided. Although

rapamycin decreases DD it never returns to baseline.

Suppl. 5A-B: P-values not shown in between the groups

As requested, we have added the p-value between all groups in (**Supplementary Fig. 6A,B and Supplementary Fig. 8B,C** in the new submission). Throughout the manuscript, to simplify and increase the clarity of the figures, we added p-values only between the groups that are discussed in the manuscript. All raw data will be provided with the paper as “Source Data” file.

Reviewer #3:

We thank the reviewer very much for the constructive comments and suggestions that have significantly improved and strengthen the quality of the manuscript.

1. In line 27 and figure 4F, the conclusion that oocyte failure to repair damaged DNA is due to the inability of DDR proteins to access altered “closed” chromatin conformation in DNA-damaged oocytes cannot be drawn by listing only one DDR protein, RAD51.

Thank you very much for this valuable comment, to which we completely agree. As suggested, we have investigated Ku80, a recently identified NHEJ repair protein in full-grown oocytes (PMID: 37407587). Similar to RAD51, Ku80 localization to the DNA was not increased significantly following DNA damage, whereas inducing autophagy by rapamycin (by not by rapamycin+spautin-1) increased Ku80 localization to the DNA. We have added these new data in **Supplementary Fig. 5A,B**. These results further support our conclusion that DNA repair proteins, at least RAD51 and Ku80 which are required of HR and NHEJ, respectively, cannot access the damaged DNA in fully grown oocytes.

2. In line 279, the colocalization of RAD51 and DNA is verified by only one method. It can also be validated by co-immunoprecipitation (ChIP or CUT&Tag) combined with PCR.

Thank you very much for this valuable suggestion. To our knowledge, ChIP and CUT&Tag techniques are not reported in any previous studies involving mammalian oocytes, due to the large number of cells needed to carry out these techniques. Moreover, PCR following chromatin isolation is not feasible because the sites of DNA damage are very random and unpredictable, making primer design challenging. Alternatively, we were able to isolate DNA-protein complex using chromatin extraction protocol followed by western blot analysis of RAD51. Similar to our immunocytochemistry data, we found that RAD51 protein levels in the extracted chromatin did not vary significantly between control and etoposide-treated oocytes. Importantly, autophagy induction with rapamycin treatment, but not with rapamycin+spautin-1, significantly increased RAD51 levels in the extracted chromatin of etoposide-treated oocytes, further confirming that RAD51 localization to the DNA is impaired in DNA-damaged oocytes. We have added these new data in **Fig. 3F**.

3. As shown in the figure 2A-D, it indicates that there is a certain autophagy activity in oocytes, but the level of autophagy in oocytes after etoposide treatment decreases, Whether the DNA damage caused by this treatment is too severe, autophagy activity is reduced due to activation of the apoptosis pathway. Is it possible to detect apoptotic activity in oocytes after etoposide treatment?

Thank you very much for this suggestion. As suggested, we have examined caspase 3 activity, a marker of apoptosis, and we did not find a significant difference between control and etoposide-treated oocytes (**Supplementary Figure 1B**), indicating that

treating the oocytes with etoposide, at least at the used concentration, do not induce apoptosis. This finding is consistent with our and others' observations, indicating that etoposide-treated oocytes are viable and resume meiosis even at a higher concentration (100 μ g/ml) than the one which we used in this study (50 μ g/ml). Supplementary Movie 2 shows an example of an etoposide-treated oocyte that completes meiosis I and extrudes the first polar body (~10 h after DNA damage induction).

4. The article writes that mouse and porcine oocytes fail to activate autophagy in response to DNA damage, is there no change of γ H2AX levels in aging oocytes or after etoposide-treated oocytes treated with spautin-1? In line 233, the result shown in the Supplementary Figure 2I-K seems contradictory to this view, suggests that DNA damage can induce autophagy in oocytes.

Thank you very much for this important observation, to which we agree. We apologize if our writing was vague, and we totally agree with the reviewer's observation. We believe that autophagy plays a role in DNA damage repair and that fully grown oocytes contain certain activity of autophagy (but not devoid of autophagy, as shown in control oocytes in **Fig. 2A,C**). This basal level of autophagy is likely protecting the oocyte against the mild threatening by endogenous DNA assaults. So, upon the exposure to moderate DNA damage (as in our study), it is expected that this basal level of autophagy available in the oocyte is consumed in response to initial DNA damage. However, the oocyte fails to activate additional autophagy, leading to reduced autophagy after DNA damage. This is supported by our data where autophagy inhibition by spautin-1 increased the level of DNA damage and further increased the level of DNA damage in etoposide-treated oocytes (**Fig. 2K-L and Fig. 2G,H**). Together, our data suggest a model that the full-grown oocyte contains a certain basal level of autophagy which is consumed in the initial response to DNA damage. However, due to the failure to further activate autophagy, the oocyte has a limited capacity to repair moderate/severe DNA damage. This model is supported by our data that autophagy induction promotes DNA damage repair following DNA damage. We have added this model to the text in **lines 485-495**.

5. In line 390, the article writes that p53-mediated apoptosis is not functional in fully grown GV oocytes, but the cited literature identifies that p63 regulates the apoptosis pathway of GV oocytes, and the way described here is ambiguous, making people mistakenly believe that there is no apoptotic pathway in oocytes.

We apologize if our language was vague. Suh et al. (PMID: 17122775108) found that, in response to DNA damage, p53-null oocytes were very similar to that of wild-type controls (Fig. 2d), indicating that p53-mediated apoptosis is not functional in DNA damaged oocytes. In the same paper, the authors found that although p63 regulates apoptosis in "non-grown and growing" GV oocytes (in primordial and primary follicles), p63 expression was lost in "full-grown" GV oocytes (within follicles recruited for ovulation, the stage which we study in our manuscript). Therefore, p63-mediated

apoptosis is also not functional in fully grown GV oocytes. We have modified the text to read, “For poorly understood reasons, the DNA damage response is not robust, and both p53- and p63-mediated apoptosis are not functional in fully grown GV oocytes” (line 461).

6. In line 289 and figure 4F, the relationship between RAD51 localization and chromatin conformational changes is correlated without direct evidence.

In the revised version, we have included new experiments to manipulate the chromatin status using 3 different approaches (increasing open chromatin state by HDAC inhibition and increasing closed chromatin state by HAT or PARP-1 inhibition) to confirm our conclusion that, “opened chromatin conformation is required for efficient RAD51 localization to damaged DNA sites”:

Histone acetylation is a major regulator for chromatin status and largely regulated by histone deacetylases (HDACs) and histone acetyl transferases (HATs). Therefore, HAT inhibitors have been shown to induce “closed chromatin” status, whereas HDAC inhibitors have been shown to induce “open chromatin” status (PMID: 28625481; PMID: 15292402; PMID: 31799598; PMID: 33875841).

1. Our data indicate that the chromatin is relatively closed in DNA damaged oocytes (**Fig. 4A-C; Fig. 4D,E and Supplementary Fig. 6**) with decreased RAD51 localization at the DNA (**Fig. 3**). To confirm that “closed chromatin” conformation is the cause, at least in part, of decreased RAD51 localization to the DNA in DNA-damaged oocytes, we increased “open chromatin” status using HDAC inhibitor, trichostatin A, in DNA-damaged oocytes and examined RAD51 localization. We found that increasing open chromatin status, by trichostatin A, increased RAD51 localization to the DNA in etoposide-treated oocytes, indicating that “closed chromatin” structure is the cause, at least in part, of decreased RAD51 localization to the DNA in DNA-damaged oocytes. We have added this new data in **Supplementary Fig. 7A,B**.

2. We found that, following DNA damage, the chromatin is relatively closed with reduced RAD51 localization to the DNA and autophagy induction (by rapamycin treatment but not by rapamycin+spautin-1) increased the level of open chromatin state and increased RAD51 localization to the DNA in DNA-damaged oocytes. Importantly, treating the oocytes with garcinol, an HAT inhibitor that has been shown to increase “closed chromatin” structure, decreased RAD51 localization to the DNA in rapamycin-treated DNA-damaged oocytes, indicating that autophagy increased RAD51 localization to the DNA, at least in part, by promoting “open chromatin” structure. We have added this new data in **Fig. 3A,B**.

3. PARP-1 regulates chromatin remodeling, and its activation promotes PARylation and “open chromatin” structure (PMID: 28676700; PMID: 34432161). We found that inducing more “closed chromatin” structure by inhibiting PARP1 abolished the “open chromatin status” in rapamycin-treated DNA-damaged oocytes (**Fig. 5C-E**) and

importantly, impaired RAD51 localization to the DNA (new data, **Fig. 5F,H**), indicating that autophagy promotes RAD51 localization to the DNA, at least in part, by PARP-1-mediated “open chromatin” status.

Importantly, our findings are consistent with previous publications (in non-oocyte models) that open chromatin status is required for DNA repair protein accessibility to damaged DNA (PMID: 24674626; PMID: 17893239; PMID: 11003660; PMID: 16936822; PMID: 25250043).

7. In line 294, you check chromatin status with alkaline comet assay (DNase sensitivity assay). Can you supplement the ATAC-seq experiment for verification?

Thank you very much for this valuable suggestion. We contacted several companies and core facilities to prepare the DNA libraries for bulk ATAC-seq. However, their responses are always “typically the minimum starting number of cells for the bulk ATAC prep is 50,000 cells”. This is an impossible number of cells in our system where one mouse yields ~40-60 oocytes. We have contacted several experts in the field, and we already did several trials to optimize the protocol in our laboratory using low numbers of oocytes. Although we believe that we achieved a lot of progress, we are still not able to establish a stable protocol. However, we supported our DNase sensitivity assay conclusions using acH3K9/14, a marker for open chromatin status (**Fig. 4D,E and Supplementary Fig. 6E,F**), and got very similar results to the DNase sensitivity assay experiments. We feel that optimizing this technique in the oocyte system is outside the scope of this study and will take a lot of time. Given that the DNase I sensitivity assay is a reliable and accurate method to assess the state of chromatin in mammalian cells (PMID: 26605532; PMID: 8047169; PMID: 22955617), we hope that the reviewer agrees with us that using acH3K9/14 marker and DNase sensitivity assay are sufficient to support our conclusion,

8. The view that induction autophagy repair oocyte DNA damage is more one-sided for the improvement of overall oocyte quality, and excessive autophagy levels may reduce oocyte quality by affecting cytoplasmic maturation, when DNA repair is not meaningful, in other words, induction of autophagy may not be the best way to repair oocyte DNA damage.

Thank you very much for this comment, to which we completely agree. We have used rapamycin to induce autophagy. Rapamycin is an FDA approved drug that has been shown to improve the longevity in humans. Moreover, it has been shown that autophagy induction by rapamycin treatment improves the quality of mammalian oocytes and embryos (i.e. increased oocyte maturation rates and the percentage of embryos reaching the blastocyst stage). However, we understand the reviewer’s concern and modified our language in the discussion section to read, “However, future research is essential to determine whether excessive autophagy induction affects other cytoplasmic events in mammalian oocytes” (**lines 480-481**).

We thank the reviewers and appreciate the careful review given to our manuscript. We have extensively addressed and clarified the concerns of the reviewers.

Below are point-by-point responses.

Reviewer comments:

Reviewer #1:

In response to my comments, and those of the other reviewers, the authors have provided additional controls and new experiments to support their existing data. I still find that the wording and conclusions drawn are too strong in context of the data presented. In particular, the main concept that aging-related aneuploidy is the result of reduced autophagy leading to a weakening of the DNA damage response and increased unrepaired DNA damage was not directly demonstrated.

We thank the reviewer for this excellent comment. Although linking reduced autophagy and aging-related aneuploidy was not one of our focus in the previous submission, we agree with the reviewer and find this point is very important to be addressed.

- 1- In the previous version, we found that autophagy level is significantly reduced in oocytes of aged mice when compared to those collected from young mice (**Suppl. Fig. 9A,B**).
- 2- Similar to previous publications, we found that aged oocytes (which have reduced autophagy activity) exhibit higher levels of DNA DSBs (g-H2AX signals, **Suppl. Fig. 9C,D**).
- 3- To investigate whether reduced autophagy contributes to increased DSBs in maternally aged oocytes, we induced autophagy in maternally aged oocytes and assessed DSBs. We found that autophagy induction by rapamycin significantly decreased DNA DSBs. Importantly, autophagy inhibition by spautin-1 abolished the rescue effect of rapamycin on DNA DSBs in maternally aged oocytes (**Suppl. Fig. 9C,D**), indicating that *reduced autophagy contributes to the elevated DNA damage in maternally aged oocytes*.
- 4- In the current revised version, we have added new data showing that, similar to previous publications, maternally aged oocytes have higher rates of aneuploidy. To investigate whether reduced autophagy contributes to increased aneuploidy incidence in maternally aged oocytes, we induced autophagy in maternally aged oocytes and assessed aneuploidy. Importantly, autophagy induction by rapamycin decreased the incidence of aneuploidy in aged oocytes (**Suppl. Fig. 9E,F**). To confirm that rapamycin decreases the incidence of aneuploidy in maternally aged oocytes by stimulating autophagy, we included another spautin-1 group and showed that autophagy inhibition by spautin-1 abolished the partial rescue effect of rapamycin on aneuploidy in maternally aged oocytes, indicating that reduced autophagy contributes to increased aneuploidy incidence in maternally aged oocytes. We have added these new data in **Suppl. Fig. 9E,F**.

- 5- Moreover, we also showed that DNA damage in oocytes leads to aneuploidy (Fig. 1G-J).
- 6- Taken together and given that maternally aged oocytes harbor high levels of DNA damage, our data indicate that reduced autophagy leading to elevated DNA damage is a contributing factor to the increased aneuploidy incidence in maternally aged oocytes.

However, we have tempered our language and did not build a direct conclusion that aging-related aneuploidy is **a result** of reduced autophagy. Instead, we mentioned “Taken together, our results show that reduced autophagy is a significant contributor to the further weakened DDR in oocytes from aged mice, and that autophagy induction represents a promising approach to ameliorating DNA damage-induced alterations in oocytes from aged females” in **lines 456-459**.

Reviewer #2:

1- The authors have responded to some of the comments by additional experiments. The paper reports interesting results particularly regarding the effects of chromatin structure in the accessibility to double strand break repair factors in GV oocytes. However, the authors overstate their results in a misleading way ultimately compromising their credibility as pointed out before and again explained below. For example, the authors indirectly induce DNA damage via the topoisomerase II inhibitor etoposide and indirectly assess DNA double strand break sites by gH2AX and then generalize etoposide treatment and gH2AX levels to all “DNA damage”. This is misleading because of the wide variety of DNA damage types and repair mechanisms. In the case of oocytes the type of damage and outcome is of central importance because oocytes are the predominant source of aneuploidy but have only a very minor contribution to germline mutations. Therefore, precision of language when stating scientific results is an important part of scholarly publishing. Instead the authors directly insert their interpretations into the results.

Thank you for this comment. In fact, the precision of our language and being accurate is the reason why we used the term “DNA damage” but not “DNA double strand breaks” when describing gH2AX results. Although H2AX is phosphorylated after DNA double-strand breaks, recent data suggest that gH2AX is also increased following single-strand breaks <https://www.nature.com/articles/ncomms15104>; <https://www.nature.com/articles/cdd201253>.

We used the term “DNA damage” instead of DSBs for this reason, but not because we wanted to generalize etoposide treatment and gH2AX levels to all “DNA damage. In fact, mentioning DSB would increase the significance of our manuscript, given that it is the most lethal form of DNA damage. It is of note that, in this manuscript, we did not only use gH2AX, but we confirmed the DNA damage by comet assay and the observation of DNA fragmentation in live and fixed metaphase I oocytes.

We also used 3 different forms of DNA damage inducers (UV, ionizing radiation and etoposide). Although all these treatments induce double strand breaks and DNA fragmentation in mouse oocytes, they can induce other types of DNA damage.

Determining the extent of these treatments to induce different types of DNA damage is beyond the scope of this study. Importantly, all treatments and assays provided consistent results.

Given that DNA double strand breaks and single strand breaks belong to “DNA damage”, we mentioned in the previous manuscript version, “DNA damage breaks (hereafter referred to as DNA damage)”. Moreover, we used the term “DNA damage” to be consistent with the common language in the oocyte field which uses the term “DNA damage” after assessing etoposide treatment and γ H2AX levels. For example,

Marangos and Carroll

(<https://www.sciencedirect.com/science/article/pii/S0960982212003909>) mentioned “etoposide (5 μ g/ml for 3 hr) causes DNA damage as evidenced by the presence of phosphorylated H2AX at Ser139 (γ H2AX)”

<https://www.nature.com/articles/ncomms9706>: mentioned, “DNA damage induced in prophase-arrested oocytes persists through to MI as demonstrated by γ H2AX labelling”.

Cai et al. (<https://www.nature.com/articles/s41419-023-05886-x>) mentioned “the extent of etoposide-induced DNA damage or the time taken for repair (as determined by loss of γ H2AX labeling)”.

Rémillard-Labrosse et al. ([https://www.fertstert.org/article/S0015-0282\(19\)32681-0/fulltext](https://www.fertstert.org/article/S0015-0282(19)32681-0/fulltext)) mentioned, “the chromatin in those etoposide-treated oocytes that extruded a polar body was indeed substantially damaged as evidenced by intense γ H2AX labeling”.

We are confused by the reviewer’s concern that “oocytes have only a very minor contribution to germline mutations” and how this relates to our findings. First, although we agree that maternal contribution is less than paternal, but it is not “very minor”. Second, even if the maternal contribution is less than paternal contribution, this does not mean that it is not significant. Third, this study examined only full-grown oocytes (the pool of oocytes which will be recruited for ovulation). Given that this pool is a small fraction of total oocytes in the ovary, the mutations present in full-grown oocytes would not significantly influence the overall mutation rate in all oocytes within the ovary but would have significant consequences on future embryo development after fertilization. Fourth, we showed in the manuscript that inducing DNA damage leads to aneuploidy. It is already known that the consequences of aneuploidy are very unpredictable and depend on the loss/gain genes. These consequences could be fertilization failure, early embryo arrest, failure in blastocyst formation, implantation failure, early embryonic loss, abortion or even the birth of fetal congenital abnormalities. Therefore, it is very plausible that most of DNA damage-induced mutations cause embryonic death before transmitting these mutations to the new generations. Finally, Goldmann et al. (<https://www.nature.com/articles/s41588-018-0071-6>) demonstrated that “Germline de novo mutation clusters arise during oocyte aging in genomic regions with high double-strand-break incidence and Gao et al.

(<https://www.pnas.org/doi/full/10.1073/pnas.1901259116>) entitled “Overlooked roles of DNA damage and maternal age in generating human germline mutations.

However, as suggested, we have now replaced the term “DNA damage” by “DNA double strand breaks” throughout the manuscript.

2- The issue of their misleading overstatements is continued into the new results. As already pointed out before, rapamycin has multiple effects and how it relates to the observed induction of autophagy is unclear given the numerous other targets it has. The authors now use spautin-1, which has been linked to autophagy inhibition by inhibiting the ubiquitin-specific peptidase USP10. Instead of stating what spautin-1 really targets, the authors superficially (in a strict sense falsely) state “Spautin-1 is a specific and potent small molecule inhibitor that efficiently inhibits autophagy by degrading Beclin-1”. As usual with chemical inhibitors, however, there is no unique specificity and instead it was recently published to also inhibit the mitochondrial complex I.

We kindly wish to highlight that we did not revise our description of spautin-1 in the previous revised version because we did not receive this comment in the first round of revision, despite the description of spautin-1 in the previous revised version was unchanged from that of the initial manuscript. Moreover, the use of spautin-1 to inhibit autophagy in all rapamycin experiments was a valuable and important suggestion from Reviewer 1.

Spautin-1 (**Specific Potent Autophagy Inhibitor 1**) has been described/used in several publications as a specific inhibitor for autophagy ([https://www.cell.com/cell/fulltext/S0092-8674\(11\)01007-5](https://www.cell.com/cell/fulltext/S0092-8674(11)01007-5); <https://www.nature.com/articles/s41467-023-38318-7>; <https://www.nature.com/articles/nature16063>; <https://www.nature.com/articles/s41467-017-02536-7>).

We agree that Kunimasa et al. (Scientific Reports 2022) showed that spautin-1 inhibits the mitochondrial complex I. However, autophagy inhibition (even with other different autophagy inhibitors) decreases mitochondrial complex I (PMID: 25325275; PMID: 27889640) which raises the possibility that decreased mitochondrial complex I in spautin-1-treated cells could be a consequence of autophagy inhibition (the possibility that not “directly” examined yet).

Importantly, as the reviewer knows, mitochondrial complex I is eliminated in oocytes (Rodríguez-Nuevo et al. Nature. 2022 (PMID: 35859172). Thus, there is no chance for spautin-1 to affect mitochondrial complex I in our study. Similarly, IKZF1 data is controversial as it is also increased following autophagy inhibition by 3-MA (<https://www.sciencedirect.com/science/article/pii/S0006497119332823>) and IKZF1 has undocumented role in mammalian oocytes. Therefore, to our knowledge, there is no direct and “reproducible” results confirming the lack of spautin-1 specificity in oocytes.

However, we agree that chemical inhibitors have always the concern of specificity. That is why we did not use a single inhibitor to build our conclusion. Instead, we used different drugs to induce/inhibit autophagy and examined their effects on DNA damage, seeking for consistent results. All of spautin-1, rapamycin and MG-132 gave consistent results. Moreover, as suggested below, we now included new results of ATG5 knockout oocytes (i.e. showing increased levels of DNA DSBs and sensitivity to DNA damage in ATG5 KO oocyte when compared to WT oocytes), providing a direct link between autophagy and DNA DSBs (**Fig. 3**). Importantly, rapamycin treatment significantly decreased etoposide-induced DSBs in WT oocytes, but not in Atg5^{-/-} oocytes (**Fig. 3C,D**). This result indicates that rapamycin promotes DDR through autophagy induction (but not through other mTOR-dependent pathway), making it a valuable tool for assessing the impact of autophagy induction on DNA damage within our experimental system.

We have also modified our language in the revised version (deleting the word “specific” and added more detail about spautin-1 target) to read, “Spautin-1 is a potent small molecule inhibitor that efficiently inhibits autophagy by inhibiting ubiquitin-specific peptidases, USP10 and USP13, leading to Beclin-1 degradation”.

3- To support their claims, the authors then use MG-132 and claim it as autophagy inhibitor, which again is at least a gross oversimplification because MG-132 is an inhibitor of the UPS proteasome, which while playing also a role in autophagy is a distinct part of the proteostasis network. Instead, the authors mislead again by stating “Proteasomal inhibitor, MG-132, is known to induce autophagy by increasing LC3-I to LC3-II conversion”. While that may be a consequence of proteasome inhibition, there are multiple other consequences. Therefore, the authors’ far reaching claim of a specific role of autophagy in the observed effects is insufficiently supported.

We did not claim that MG-132 is an autophagy inhibitor, instead we used it to induce autophagy through rapamycin-mTOR independent pathway. What we stated, “Proteasomal inhibitor, MG-132, is known to induce autophagy by increasing LC3-I to LC3-II conversion”. The words “proteasomal inhibitor” indicate clearly that its effect on autophagy is indirect and we never mentioned that MG-132 is a specific autophagy inducer. We would like to highlight that our description of MG-132 in the revised version is unchanged from that in the first version of the manuscript.

However, we have now modified the sentence and tempered our language to read, “Proteasomal inhibitor, MG-132, is also known to induce autophagy “indirectly” by inhibiting the proteasome degradation system, leading ultimately to increased LC3-I to LC3-II conversion”.

4- The new results from the atg5 knockout mice they include in their response (but not in the manuscript) could shed light on this and could indeed overcome the limitations of the current study that relies exclusively on inhibitors (etoposide, rapamycin, spautin-1,

MG132) all of which have the issue that their usage cannot support the claims on specificity that the authors put forward.

We agree with the reviewer comment regarding the use of ATG5 KO data. As suggested, we have now included the results of ATG5 knockout (KO) oocytes in the revised version. We have added new data that (1) Atg5 KO oocytes have reduced levels of autophagy (**Fig. 3A,B**) and (2) showing that Atg5 KO oocytes exhibited higher levels of γH2AX when compared to WT oocytes (**Fig. 3C,D**). Moreover, compared to WT oocytes, Atg5 KO oocytes were sensitive to DNA damage induction and showed a significantly higher levels of γH2AX following the exposure to a low concentration (10 μg/ml) of etoposide treatment (**Fig. 3C,D**). These results support our chemical inhibitor results (rapamycin, MG-132 and spautin-1) and confirms that autophagy and DNA DSBs are directly linked.

Importantly, we found that rapamycin treatment significantly decreased etoposide-induced DSBs in WT oocytes, but not in Atg5^{-/-} oocytes (**Fig. 3C,D**). This result indicates that rapamycin promotes DDR through autophagy induction (but not through other mTOR-dependent pathway), making it a valuable tool for assessing the impact of autophagy induction on DNA damage within our experimental system. We have added these data in **new Fig. 3**.

5- The misleading overstatements by the authors are found throughout the manuscript and compromise the scientific value of their work. Another typical example "...ATM kinase, the master regulator of DNA damage checkpoint... Therefore, phospho-ATM (p-ATM) is considered a sensitive marker of DNA damage sensing and repair." belongs in an opinion paper not in a research article as there are distinct DNA damage checkpoints some of which are regulated by ATM and ATM is involved e.g. in responding to DSBs but other damage types do not involve ATM.

We kindly wish to highlight that we did not revise our description of ATM in the previous revised version because we did not receive this comment in the first round of revision, despite the ATM description in the previous revised version was unchanged from that of the initial manuscript.

The description of ATM as the master regulator of DNA damage checkpoint/response was stated in several papers (PMID: 29317520; PMID: 34429537; PMID: 33239428). **However, we have now deleted these words.**

We respectfully disagree that "ATM is involved in responding to DSBs but other damage types do not involve ATM". In fact, the precision of our language is the reason why we used the term "DNA damage" and not "DSBs" when describing ATM role. Although ATM is involved in DSBs, there are recent data suggesting that ATM is also involved in the response to single strand breaks

(<https://www.pnas.org/doi/full/10.1073/pnas.1416031112>).

However, we have now modified the sentence to read, "Upon DNA DSBs, ATM kinase, is activated, a necessary step for DDR initiation. When DSBs are repaired, ATM is inactivated" in lines 305-306.

Reviewer #3 (Remarks to the Author):

The authors have improved the manuscripts. However, similar to before, many conclusions still lack direct evidence, most conclusions drawn in your manuscript are derived from immunofluorescence experiments. For example, many published papers (PMID: 29720659; PMID: 28723896; PMID: 32169215) have showed the ATAC-seq to check chromatin status. The experiment is feasible in oocytes and very important to support the conclusion. However, the authors use DNase sensitivity assay and acH3K9/14 to check chromatin status. I think the results were indirect.

Thank you very much for raising this comment.

Immunofluorescence experiments: Due to the limited number of biological materials in mammalian oocytes, fluorescence microscopy are widely used as the only or the major technique to provide robust results as exemplified by many publications (<https://www.nature.com/articles/s41586-023-06700-6> ; <https://www.nature.com/articles/s41467-020-15470-y#Sec10> and <https://www.nature.com/articles/ncomms8217#Sec9>).

However, in our manuscript, we complemented our immunofluorescence results by several other techniques such as western blot analysis (Fig. 1C; 2C,D; Suppl. Fig. 2B; Suppl. Fig. 8A), chromatin extraction (Fig. 4F), time-lapse live imaging (Fig. 1G,H) and comet assay (Fig. 1D-F; 5A-C; 6C-E; Suppl. Fig. 6A-D; Suppl. Fig. 8B,C).

ATAC-seq: Although a few labs were able to make ATAC-seq works with a very low oocyte number, the reproducibility is still a big issue. We collaborated with an expert, and we did several trials and sent many samples for sequencing, but the quality of samples (due to decreased cell numbers) was always an issue to get stable results. Communications with several university cores and leading companies in ATAC-seq analysis indicate that it is not technically feasible at this time.

Just examples of some leading companies' webpages:

Active motif company (<https://www.activemotif.com/catalog/1233/atac-seq-service>) requires, "ATAC-Seq Services Genome-wide profiles of open chromatin regions from **as few as 50,000 cells**".

CD genomics (https://rna.cd-genomics.com/atac-sequencing.html?gad_source=1&gclid=CjwKCAjwrvyxBhAbEiwAEg_KguXa8xHNsP950LPuS0NH2Vop-xzvj4xVGu2R4TizHsL0poTd4wU5SB0Cs7UQAvD_BwE) requires "Cell sample $\geq$ 50,000 cells; Nucleus sample $\geq$ **50,000 nuclei**".

GENEWIZ (https://web.genewiz.com/atac-seq-faq?_ga=2.259071939.811806469.1715496735-1052191642.1715496735) requires “Minimum Amount: **3 tubes of 100,000 cells in 500 µL each**”.

Novogene (<https://www.novogene.com/us-en/services/research-services/epigenome-sequencing/atac-seq-assay-for-transposase-accessible-chromatin-with-sequencing/>) requires **≥50,000 cells**.

Li et al. (<https://star-protocols.cell.com/protocols/968>) described “Low-input ATAC&mRNA-seq protocol suitable for limited numbers of cells ranging from **5,000 to 20,000 cells**”.

Unfortunately, these are impossible and infeasible numbers in our system where one mouse yields ~40-60 oocytes.

DNase I hypersensitivity assay has been extensively used for decades to assess chromatin state. Several studies have compared sequencing after DNase I treatment with ATAC-seq (sequencing after Tn5 transposase treatment) and found similar and comparable results in detecting open chromatin regions without any difference in efficiency (e.g., PMID: 24097267; PMID: 31584093; PMID: 28846090; PMID: 25559105).

Although we understand that H3K27me3 is not a direct evidence, but it is an epigenetic mark that associates with Heterochromatin. Consistent with DNase sensitivity assay and H3K9/14ac, we found that H3K27me3 levels were significantly increased in etoposide-treated oocytes and significantly decreased following autophagy induction. We have added these **new data in Fig 5F,G**.

Finally, we would like to clarify that the information that would be gained with ATAC-seq analysis would not further strengthen our conclusions because the DNA damage sites are random and unpredictable so identifying the specific locations of open/closed chromatin is not informative. Our primary focus is to determine whether chromatin is more open or closed following treatments. Therefore, to answer this question (i.e. the chromatin is more open or closed), we believe that, similar to ATAC-seq, DNase sensitivity assay (supported by H3K9/14ac and H3K27me3) provides direct evidence about chromatin state.

We thank the reviewers and appreciate the careful review given to our manuscript.

Below are point-by-point responses.

Reviewer comments:

Reviewer #1:

Thank you for including additional data in the manuscript. However, as raised in previous reviews, the wording of the manuscript and conclusions drawn are too strong throughout based on the data presented.

Thank you for pointing this out. As requested, we have toned down our language and rewrote the text in several places. For example, we have edited the abstract, deleted a few sentences and modified several sentences (highlighted in red in the revised manuscript).

Reviewer #2:

The authors have adequately addressed my comments. They have now more precisely described their results and included data on the Atg5 knockout mice that strengthen their conclusions.

Thank you very much.

Reviewer #3:

I still think that the methods using DNase sensitivity assay and histone modification to check chromatin status were indirect. Many published papers (PMID: 29720659; PMID: 28723896; PMID: 32169215) have showed the ATAC-seq to check chromatin status in hundreds mouse oocytes and embryos.

We respectfully disagree that DNase sensitivity assay is an indirect method for assessing chromatin status. We would like to clarify that both DNase I sensitivity assay and ATAC-seq are based on the same principle for assessing chromatin status. Both assays depend on the accessibility of DNase I or Tn5 transposase to “open” chromatin sites. That is why the DNase I sensitivity assay has been extensively used for decades to assess chromatin state and several studies reported similar results in detecting open chromatin status without any significant difference in efficiency between the two assays (e.g., PMID: 24097267; PMID: 31584093; PMID: 28846090; PMID: 25559105).

ATAC-seq has the advantage of determining the “exact sites” of open and closed chromatin. However, since DNA DSB sites in our study are random and unpredictable, identifying these specific sites is not relevant. In addition, as mentioned in my previous response, the ATAC-seq technique requires a large number of cells (according to several leading companies and experts in the field) to yield reliable results. Therefore, we believe that the DNase sensitivity assay is more accurate for determining the chromatin status in our system.