EMBO reports

Characterization of histone inheritance patterns in the *Drosophila* female germline

Elizabeth Kahney, Emily Zion, Lydia Sohn, Kayla Viets-Layng, Robert Johnston and Xin Chen
DOI: 10.15252/embr.202051530

Corresponding author(s): Xin Chen (xchen32@jhu.edu)

Review Timeline:

Submission Date:	17th Aug 20
Editorial Decision:	9th Oct 20
Revision Received:	1st Feb 21
Editorial Decision:	8th Mar 21
Revision Received:	2nd Apr 21
Accepted:	16th Apr 21

Editor: Esther Schnapp

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision 9th Oct 2020

Dear Xin,

Thank you for your patience while your manuscript was peer-reviewed at EMBO reports. We have finally received the full set of referee reports that is pasted below.

As you will see, all referees acknowledge the overall quality of your work and that the findings are potentially interesting. While referee 2 is most positive, referees 1 and 3 each raise several concerns about the conclusiveness of the data and make suggestions for how the study could be strengthened. I am aware that they together suggest a substantial amount of revisions, and I am happy to discuss the revisions over the phone or video chat with you. You might also already be able to address some of the points.

I would thus like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of major revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss this further. You can either publish the study as a short report or as a full article. For short reports, the revised manuscript should not exceed 27,000 characters (including spaces but excluding materials & methods and references) and 5 main plus 5 expanded view figures. The results and discussion sections must further be combined, which will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. For a normal article there are no length limitations, but it should have more than 5 main figures and the results and discussion sections must be separate.

Regarding data quantification, please specify the number "n" for how many independent experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. This information must be provided in the figure legends. Please also include scale bars in all microscopy images.

IMPORTANT NOTE: we perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:
1) A data availability section providing access to data deposited in public databases is missing. If you have not deposited any data, please add a sentence to the data availability section that explains that.
2) Your manuscript contains statistics and error bars based on n=2. Please use scatter blots in these cases. No statistics should be calculated if n=2.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).
See https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf for more info on how to prepare your figures.

3) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: <https://www.embopress.org/page/journal/14693178/authorguide#expandedview>

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

4) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

5) a complete author checklist, which you can download from our author guidelines <https://www.embopress.org/page/journal/14693178/authorguide>. Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

6) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (<https://orcid.org/>). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines <https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines>

7) Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database (see https://www.embopress.org/page/journal/14693178/authorguide#datadeposition). Please remember to provide a reviewer password if the datasets are not yet public. The accession numbers and database should be listed in a formal "Data Availability" section placed after Materials & Method (see also https://www.embopress.org/page/journal/14693178/authorguide#datadeposition). Please note that the Data Availability Section is restricted to new primary data that are part of this study. * Note - All links should resolve to a page where the data can be accessed. *
If your study has not produced novel datasets, please mention this fact in the Data Availability Section.

8) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and

instruction on how to label the files are available at
<https://www.embopress.org/page/journal/14693178/authorguide#sourcedata>.

9) Our journal also encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at https://www.embopress.org/page/journal/14693178/authorguide#referencesformat

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File (RPF) to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Kind regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

Summary:

This paper aims to understand how old and new histones are partitioned in asymmetrically dividing stem cells. The authors propose a model in which old and new H3-H4 tetramers are inherited in an asymmetric manner. Using a sophisticated switchable tagging system to label old and new histones, the authors test this model in asymmetrically dividing germ line stem cells (GSCs) that reside in the Drosophila ovary. The authors find that new and old histones are differentially distributed on large regions of GSC chromosomes, examined at prophase and anaphase. This is a striking result, which appears to be specific to histone H3, and to GSCs and daughter CB cells (Figures 1 and 2). Although this labelling system has been developed and used by this laboratory in

past publications, a few aspects need to be clarified for these particular set of experiments (see point 1 below). In addition, the authors test the model that the inheritance of old H3-H4 tetramers and the deposition of new H3-H4 tetramers at different sites could be responsible for differential gene expression in GSCs. For this they perform experiments correlating the localisation of stem and differentiation genes (using DNA oligopaints) with old and new histone signals (fluorescent intensity) at prophase, and at anaphase (Figures 3 and 4). The authors measure more old H3 association with dad and bam gene loci in GSCs. This is an interesting result, however it requires further validation before publication (see point 4 below).

1.Comments related to Figure 1

To label old and new histones, the authors use a heat shock controlled, dual fluorescent tag-switching histone transgene driven by nanos-Gal4 expressed in GSCs. This method allows the tagging and labelling of old histones with GFP and new histones with mCherry in cell types expressing nanos. The authors carry out the heat shock when GSCs are mostly likely in G2 phase, and analyse GSCs 34-40 hours later, at mitosis, having completed one round of DNA replication and new histone assembly. I have two questions related to this experimental set up.

My first question relates to the cell cycle regulation of histone expression (driven by nanos). Is it possible that constitutive histone expression and availability of H3 outside of S phase could lead to assembly not coupled to DNA replication? If so this might complicate the interpretation of results. Specifically, were differential histone inheritance patterns ever observed after shorter recovery times i.e. before the cell had passed through S phase?

My second question relates to the expression pattern of the Green-Eye-nanos-Gal4 driver. Is this driver expressed exclusively in GSCs and/or CBs, or also at later stages? This is important to clarify, perhaps using GFP reporter lines. If for example, it is expressed in the CB, could one be looking at the combined histone incorporation pattern that results from histones inherited from the GSC, and also histones that have switched in the CB. The same applies for CCs. Is nanos expressed in CCs? Can the switch also occur in these cells? Related to this point, it would be interesting to test whether any overlap between old and new signals is observed in CCs in which histone expression is driven by bam-Gal4.

Fig 1D: Previously, Xie et al 2015 showed in male GSCs that the histone PTM H3T3P distinguished pre-existing versus newly synthesised H3 at prophase. The authors should perform H3T3P staining on 'switched' female GSCs to determine whether a similar system might operate in females.
Fig 1D-K: The authors should indicate how GSCs and CCs were identified in each panel, perhaps showing an indicative example.
Fig 1G: Less 'yellow' signal is observed in the H3 Rev, but this is not reflected in the quantitation in 1L, which appears to be very similar to the value obtained for H3. Can the authors provide an explanation for this?
Fig 1 H-K: Were 4 or 8 CCs used for the quantitation? This is important with respect to the timing of 'switch' induction and number of cell divisions since new histone expression. Please clarify.

2.Comments related to Figure 2:

In Fig 2A, the authors again show a differential distribution of old and new histone H3 (i.e. blocks of red, green and yellow), this time captured in dividing GSCs at anaphase and telophase. This result in female GSCs is striking and convincing, but it differs from previous findings in Drosophila male

GSC, in which old H3 is selectively segregated to the GSC and new H3 to the GB. The authors should speculate further on why this phenomenon differs between males and females.

Fig 2A: The 'old H3' signal (green) appears to be enriched at the poles (although this pattern does not appear to be observed in the anaphase image presented in Figure 4C). On page 12, the authors attribute this pattern to old histone retention potentially at heterochromatin. Given that female GSCs enter immediately from mitosis into S phase, with a very short G1 phase, could this pattern be due to DNA replication? EdU co-staining might help to give insight into this localisation pattern.

Fig 2F-I: It is difficult to see the spec+ and spec- staining in these panels. It is not clear what the arrows and circles are indicating. This should be clarified in the legend.

Related to Fig 2K: Female GSCs also undergo symmetric divisions at a low frequency. Do the authors propose the measured symmetric histone inheritance correlates with such symmetric divisions? Please clarify.

3.Comments related to Figure 3:

To increase the number of GSCs available for analysis, the authors perform switch experiments in bam- ovaries. They again report a non-overlapping H3 distribution pattern, similar to wild type. However, the authors do not indicate the efficiency of the dual-switch system in this genetic background e.g. does nanos expression or cell cycle progression occur as expected? Moreover, how do the authors reconcile the fact that bam mutant GSCs undergo symmetric (and not asymmetric) divisions? Further validation of the switch system in this genetic background is required. At the very least, the authors should cells the number of cells within a single germarium that showed this pattern.

Fig 3F/G/H: It is difficult to see the overlap between the oligopaint signals and old or new H3 signals. The authors need to show a zoom and individual channels to understand better precisely what was quantified. Also, the authors should comment on why the four oligopaint foci were of unequal size. Is this perhaps due to different levels of chromatin condensation, which might, in turn, condense or spread H3 signals?

4.Comments related to Figure 4:

Fig 4A-D: As with Figure 3, it is difficult to see the overlap between the oligopaint signals and old or new signals. The authors need to show a zoom and individual channels to understand what was quantified in each case. In the examples shown, the red or green H3 signal appears to be very diffuse and I do wonder how different levels of chromatin condensation/compaction might influence quantitation. Have the authors tried to address this in their quantitation method?

Fig 4A: This image should show a GSC probed for dad in which all four dots are resolved. On a related note, was the two dot pattern ever observed in the bam mutant GSCs? If not, this might indicate potential cohesion defects in this genetic background.

Fig 4B: Are these the 4 dots indicated by the arrows? The arrangement looks different. Perhaps number the 4 dots for clarity.

Fig 4C/D: Given that the dad gene seems to localise closer to the poles than the bam gene, is it possible that this affects comparisons in H3 signals due to different levels of chromosome compaction? The authors should address whether chromatin compaction might affect the quantitation method. Moreover, with respect to resolution, by a very rough estimation, it is possible that each focus contains 100s or 1000s of nucleosomes. How can the authors be sure these H3 containing nucleosomes are associated with this specific gene? ChIP experiments might not be possible in these tissues, but perhaps super-resolution imaging might help to improve the resolution of this experiment.

Finally, given that the measured differences in old H3 retention between dad and bam genes is not huge (59% compared to 48%), I would request that the authors carry out the same analysis for an additional gene predicted to be on/off in GSCs (ideally located on a different chromosome) and also for a neighbouring gene that might be expected to show a similar pattern.

General comment on the proposed model: The authors propose a model by which old and new H3 are differentially inherited at stem or differentiation genes. How do they envision these specific genes are recognised at the replication fork? Do the authors propose unidirectional fork movement as observed in males, but instead at specific chromosomal loci?

Referee #2:

The manuscript by Kahney et al. addresses how old versus new histones are partitioned during asymmetric division of Drosophila ovarian germline stem cells (GSCs) and their daughter cells. This is a fundamental question in cell and developmental biology. The Chen lab previously demonstrated in Drosophila male GSCs that the old histone H3 and H4 are segregated to the daughter GSCs but the new H3 and H4 enriched in gonioblasts. In this paper, the authors examined whether such an asymmetric pattern of histone segregation is conserved in female GSCs. There are three major findings in this paper:

First, the authors described the segregation of different histones in different types germ cells during early oogenesis. They showed that the old and new H3 chromosome domains are non-overlapping, that this non-overlapping pattern is most prominent during the GSC division, still very discernable during the cystoblast division, but are not obvious during cystocyte divisions. In addition, the authors reported that the old and new H4 molecules are also differentially segregated between GSCs and cystoblasts, but to a less extent than H3. However, H2A does not show any significant pattern of differential segregation during the divisions of GSCs, cystoblasts, and cystocytes. Moreover, the authors showed that, despite the asymmetric segregation of H3 with respect to individual chromosome domains during GSC and CB divisions, this asymmetry is overall not significant at the whole genome level.

Second, the authors showed that the non-overlapping segregation of old versus new H3 molecules in bam mutant germ cells is similar to that of wildtype GSCs. This discovery implicates that the bam mutant germ cells are more similar to GSCs than cystoblasts, which helps to clarify a long-standing uncertainty in the field.

Third, the authors creatively used the Oligopaint IF-FISH method to reveal that regions displaying

differential distribution of old versus new H3 contain differentially expressed genes that function in cell fate regulation but not constitutively active or silent genes. This is an important proof-of-principle step towards deciphering the functional significance of differential histone segregation during cell division.

The discoveries reported in this paper are all fundamental to cell and developmental biology. They are based on quantitative analyses that are meticulously conducted and appropriately interpreted. These discoveries reveal the complex nature of asymmetric histone segregation with regard to both histone types and cell types that are previously unappreciated. The paper is overall well written and a pleasure to read. I recommend its publication without any major revision. Before the manuscript goes to press, I suggest a minor revision to address the following questions.

1. P5, para 2, line 5: "which can undergo ACD at their apical tips (Xie & Spradling, 2000)." The first report on ACD of female GSCs is Lin and Spradling in 1997 (Development, 124, 2463-2476) with a systematic description by Deng and Lin in 1997 (Dev. Biol. 189, 79-94).

2. P6. Para1. Lines 6-10: The first description of asymmetric CB division which would create oocyte versus nurse cell fate was by Lin and Spradling in 1995 (Developmental Genetics 16, 6-12.), and the first demonstration that asymmetric spectrosome inheritance may play a role in such a cell fate determination was by Deng and Lin in 1997 (Dev. Biol. 189, 79-94).

3. P11, para 2: The division of GSCs, CBs, and CCs are all equally asymmetric, at least with regard to spectrosome/fusome segregation, subsequent microtubule network formation, and eventual oocyte determination, with the initial CB division presumably pre-determines the oocyte fate (e.g., Lin and Spradling, 1995). Hence, the statement "it is possible that breaking the symmetry in preparation for cellular differentiation in the female germline lineage is accomplished by two steps, GSC division and CB division" is unlikely to be accurate because it is only reasonable to suggest that either all divisions break the symmetry (if the authors does not define "breaking symmetry" as a single step) or only CB division is the breaking step (if the authors define "breaking symmetry" as a single step) for oocyte determination. In either scenario, it would not be a two-step process involving just GSC and CB division.

4. The titles of the last two sections are too similar. The authors may want to rephrase the titles to emphasize more on the unique aspects of each of the two sections.

5. 5. The last two sections are lengthy and somewhat repetitive. If the authors can make these two sections more succinct, that will be helpful to readers.

Referee #3:

This report follows up on previous work from the Chen lab showing that old histones preferentially segregate into the stem cell during the asymmetric divisions of Drosophila male germline stem cells by examining whether this also occurs in the female germ line. They observe a non-overlapping distribution of old and new Histone H3 in the germline stem cell and its daughter, the cystoblast, whereas other Histones show a more uniform distribution. However, the old and new histones show no significant preferential segregation into either daughter during these divisions. The authors then hypothesize that the histones may be asymmetrically inherited on specific loci that control stemness versus differentiation and use in situ hybridisations to suggest that this might be the

case at the dad locus.

While the experiments appear to have been well done, the manuscript falls short of proving its main conclusion that old Histone H3 preferentially segregates on the copies of e.g. the dad gene inherited by the stem cell daughter. The assay using Oligopaint FISH lacks the resolution needed to assign the gene to a particular histone territory and the data are analysed in a way that amplifies small differences, so that an allele that has 51% of old H3 versus 49% of new H3 is scored the same way as an allele with 100% to 0%. The results are not striking despite this analysis method and this is further compounded by the fact that the assay cannot distinguish between the two copies of the maternal chromosome after replication and the two copies of the paternal chromosome. The authors try various tricks to make these effects seem more significant, but in my opinion they are trying too hard:

"However, the normalization scheme could potentially create a situation in which one dot has both the highest GFP and the highest mCherry signal, leading to a 2:1:1 pattern (see Materials and Methods), which is likely due to the high condensation of chromosomes in mitotic cells. If we consider both the 2:0:2 and the 2:1:1 pattern, approximately 71% of dad and 71% of bam FISH signals have a preferential association with old versus new histones. For the ~30% of signals where a preferential association was not detected with either old or new histones, it is possible that fewer labeled histones were incorporated at that genomic region, or that the chromatin was folded and condensed in such a manner that immunostaining was unable to detect it."

For example, the last sentence attempts to dismiss the nuclei that show equal segregation by claiming that they show less total signal for some other reason, but they present no evidence to support this view. They also fail to apply the same critical thinking to the nuclei that they score as showing asymmetric inheritance, which raises a concern about a bias in the analysis. I therefore think that these results need to be confirmed by another method, such as super-resolution imaging, for the conclusion to be compelling. For this reason, I think that this manuscript is too speculative for publication.

Other points:

1) The evidence for the uneven distribution of old and new Histone H3 in post S-phase nuclei is strong, but the authors consider only one possible explanation for this hypothesis, which is that the old Histone is being preferentially inherited by one strand of the DNA after replication. In my opinion, a more likely explanation is that the non-overlapping distributions of new and old Histone H3 corresponds to when in S-phase the DNA is replicated, as the new Histone gradually increases over time after the Flip from the green to the red version. This would suggest that the chromosomal territories that are predominantly labelled with new H3 are late replicating heterochromatic regions.

2) "The cellular specificity of the non-overlapping old versus new histone H3 patterns recapitulates what has been previously reported in Drosophila male GSCs, where the global asymmetric inheritance of old versus new H3 is specifically found in asymmetrically dividing GSCs but not in symmetrically dividing spermatogonial cells (Tran et al., 2012)."

While this statement is technically correct, it is very misleading. In the male germline stem cell, all of the new and old H3 segregate from each other into different daughters at division, which is not the case in the female germ line. It would be more pertinent to point out that the answer to the question "whether differential histone inheritance is a conserved feature of stem cells and/or asymmetrically dividing cells or not" is a clear no.

3) "Interestingly, a subset of GSC and CB divisions results in two daughters with biased old versus new H3 and H4 inheritance, reflected by a wide distribution of H3 and H4 compared to H2A, which has a more clustered distribution (Fig 2E, 2J)".

Since this subset turned out not to be significant on further analysis, why are they mentioned at all.

4) "Furthermore, the similarity of old versus new histone distribution patterns among WT GSCs, WT CBs, and bam mutant germ cells (data re-plotted in Fig EV3D for direct comparison) shed light on a long-held debate in the field whether bam mutant germ cells resemble more like GSCs or CB".

This analysis cannot shed light on whether bam mutant germ cells are more like GSCs or CBs, because GSCs and CBs are indistinguishable in their distributions of new and old histones.

Point-by-point Responses to Reviewers' Questions:

Referee #1:
Summary:

This paper aims to understand how old and new histones are partitioned in asymmetrically dividing stem cells. The authors propose a model in which old and new H3-H4 tetramers are inherited in an asymmetric manner. Using a sophisticated switchable tagging system to label old and new histones, the authors test this model in asymmetrically dividing germ line stem cells (GSCs) that reside in the Drosophila ovary. The authors find that new and old histones are differentially distributed on large regions of GSC chromosomes, examined at prophase and anaphase. This is a striking result, which appears to be specific to histone H3, and to GSCs and daughter CB cells (Figures 1 and 2). Although this labelling system has been developed and used by this laboratory in past publications, a few aspects need to be clarified for these particular set of experiments (see point 1 below). In addition, the authors test the model that the inheritance of old H3-H4 tetramers and the deposition of new H3-H4 tetramers at different sites could be responsible for differential gene expression in GSCs. For this they perform experiments correlating the localisation of stem and differentiation genes (using DNA oligopaints) with old and new histone signals (fluorescent intensity) at prophase, and at anaphase (Figures 3 and 4). The authors measure more old H3 association with dad and bam gene loci in GSCs. This is an interesting result, however it requires further validation before publication (see point 4 below).

1.Comments related to Figure 1

To label old and new histones, the authors use a heat shock controlled, dual fluorescent tag-switching histone transgene driven by nanos-Gal4 expressed in GSCs. This method allows the tagging and labelling of old histones with GFP and new histones with mCherry in cell types expressing nanos. The authors carry out the heat shock when GSCs are mostly likely in G2 phase, and analyse GSCs 34-40 hours later, at mitosis, having completed one round of DNA replication and new histone assembly. I have two questions related to this experimental set up. My first question relates to the cell cycle regulation of histone expression (driven by nanos). Is it possible that constitutive histone expression and availability of H3 outside of S phase could lead to assembly not coupled to DNA replication? If so this might complicate the interpretation of results. Specifically, were differential histone inheritance patterns ever observed after shorter recovery times i.e. before the cell had passed through S phase?

We now addressed this question using the *nanos-Gal4* driving dual color histone H3 transgene: We performed heat shock and then recovered for 18-20 hours (~ one cell cycle), we then examined eGFP and mCherry signals in the H3S10p-positive M-phase GSCs. These new results are now shown in EV Figure 1: At the first M phase, female GSCs mostly show old H3 signals because the new H3 are mainly incorporated during the subsequent S phase. By contrast, the replication-independent histone variant H3.3 display significant new H3.3 incorporation even at the first M phase (see current EV Figure 4).

My second question relates to the expression pattern of the Green-Eye-nanos-Gal4 driver. Is this

driver expressed exclusively in GSCs and/or CBs, or also at later stages? This is important to clarify, perhaps using GFP reporter lines.
This driver was obtained from Dr. Daniela Drummond-Barbosa Lab, which has been shown to be highly expressed in female GSCs and CBs, and used in the following publication: Laws, K.M., Sampson, L.L. & Drummond-Barbosa, D. Insulin-independent role of adiponectin receptor signaling in *Drosophila* germline stem cell maintenance. Dev Biol 399, 226-36 (2015). However, this driver is not expressed exclusively in GSCs and CBs, but in all early-staged germ cells. We added the reference and more clarification on the gene expression pattern it turns on.

If for example, it is expressed in the CB, could one be looking at the combined histone incorporation pattern that results from histones inherited from the GSC, and also histones that have switched in the CB. The same applies for CCs.
Given our heat shock regime, even if CB inherits some histone-eGFP from the first GSC division after heat shock (Figure 1B-C), it should still fit the design that histone-eGFP represents old histone and histone-mCherry represents new histone, the later are mainly incorporated during DNA replication. Since we specifically examine the subsequent M phase for old *versus* new histone distribution pattern, this scenario will not affect interpretation of the results even if it happens. (Note: The time frame we used to capture M phase cell post-heat shock would not allow enough time for the CB or CC to inherit histone-mCherry from their mother cell and use it as the old histone in the subsequent mitosis.)

Is nanos expressed in CCs? Yes.
Can the switch also occur in these cells? Yes.
Related to this point, it would be interesting to test whether any overlap between old and new signals is observed in CCs in which histone expression is driven by bam-Gal4.
As discussed above and shown in EV Figure 1, using the two distinct color to distinguish old *versus* new histones is contingent with the cell cycle progression but not on the specific germline driver. As *nanos-Gal4* does drive gene expression in CCs, using *bam-Gal4* will not change the pattern.

Fig 1D: Previously, Xie et al 2015 showed in male GSCs that the histone PTM H3T3P distinguished pre-existing versus newly synthesised H3 at prophase. The authors should perform H3T3P staining on 'switched' female GSCs to determine whether a similar system might operate in females.
The differential phosphorylation between old and new H3 is not the focus of this manuscript. And multiple mitotic histone phosphorylation marks are differentially enriched with old *versus* new H3, which has also been shown in a previous biochemistry paper (Lin et al., 2016). We have had results showing that H3S10 phosphorylation is enriched with old H3 in mitotic female germline stem cells and included in EV Figure 3.

Fig 1D-K: The authors should indicate how GSCs and CCs were identified in each panel, perhaps showing an indicative example.
We have illustrated how GSCs and CCs were identified in Figure 1A, using a combination of the location of the cells in the germarium and morphology of spectrosome (dotted structure in GSCs and CBs) versus fusome (branched structure in CCs). We added these clarifications in the Figure 1A legend, which are very standard criteria in the field.

Fig 1G: Less 'yellow' signal is observed in the H3 Rev, but this is not reflected in the quantitation in 1L, which appears to be very similar to the value obtained for H3. Can the authors provide an explanation for this?
We agree that visually they look slightly different and indeed the Spearman's rank correlation coefficient for the H3Rev line is 0.561 (n= 50), which is slightly lower than that for the H3 line at 0.567 (n= 57). But our statistical analysis did not show significant difference between them.

Fig 1 H-K: Were 4 or 8 CCs used for the quantitation? This is important with respect to the timing of 'switch' induction and number of cell divisions since new histone expression. Please clarify.
We have now only used data for 4-cell CCs in Fig.1L. These two groups are also shown separately in EV Figure 2B-C.

2.Comments related to Figure 2:

In Fig 2A, the authors again show a differential distribution of old and new histone H3 (i.e. blocks of red, green and yellow), this time captured in dividing GSCs at anaphase and telophase. This result in female GSCs is striking and convincing, but it differs from previous findings in Drosophila male GSC, in which old H3 is selectively segregated to the GSC and new H3 to the GB. The authors should speculate further on why this phenomenon differs between males and females.
This is a very important point of this manuscript that the old *versus* new H3 asymmetry could be global as in the *Drosophila* male GSCs (Tran et al., 2012) and *Drosophila* intestinal stem cells (bioRxiv, doi: https://doi.org/10.1101/2020.08.15.252403), or local as in the *Drosophila* female GSCs (shown in this manuscript) and in Wnt3a-induced mouse embryonic stem cells (Ma et al., 2020). Since different stem cell systems have distinct cellular differentiation programs and different changes in gene expression profile, we hypothesize that these differences are related to the degree of histone asymmetry displayed during asymmetric stem cell division. This point has been discussed in (Ma et al., 2020). We will also add the speculation in the revision that it is likely due to different gene expression program changes in female *versus* male germline lineages.

Fig 2A: The 'old H3' signal (green) appears to be enriched at the poles (although this pattern does not appear to be observed in the anaphase image presented in Figure 4C). On page 12, the authors attribute this pattern to old histone retention potentially at heterochromatin. Given that female GSCs enter immediately from mitosis into S phase, with a very short G1 phase, could this pattern be due to DNA replication? EdU co-staining might help to give insight into this localisation pattern.

Since this pattern is related with the progression of cell cycle and further investigation will be needed to pinpoint this association, we have removed relevant discussion of this point.

The reviewer's suggestion is totally possible, but it will need a much more in-depth study and there are technical complications to apply EdU pulse in this experiment. For example, we have tried EdU incorporation experiment, but a short EdU pulse (i.e. 30-min) did not give out

detectable signal given the long cell cycle of female GSCs (~ 24 hours). However, if we extended the pulse time to be longer, we cannot be sure whether the incorporated EdU at anaphase and telophase is due to the previous cell cycle during S-G2 phase or the subsequent cell cycle at G1-S phase.

Fig 2F-I: It is difficult to see the spec+ and spec- staining in these panels. It is not clear what the arrows and circles are indicating. This should be clarified in the legend.

We have added an inset for each panel in Figure 2F-I specifically to show the α-Spectrin signal.

Related to Fig 2K: Female GSCs also undergo symmetric divisions at a low frequency. Do the authors propose the measured symmetric histone inheritance correlates with such symmetric divisions? Please clarify.
The reviewer is insightful that female GSCs could undergo symmetric divisions at a low frequency. This could be judged by the GSC division plane: If the division is in perpendicular to the GSC-niche interface, it is considered an asymmetric division; if in parallel, it is considered a symmetric division. For our analysis using ana-telophase GSCs, we always use the GSCs with the perpendicular division orientation. However, it is hard to judge the division orientation in prophase to prometaphase GSCs. Notably, we did not find two distinct populations of female GSCs at prophase to prometaphase, suggestion that either the differential incorporation of old *versus* new H3 is independent of the division mode, as shown and discussed in (Xie et al., 2015); or the percentage of symmetric GSC divisions is low. We can add this clarification if needed.

3.Comments related to Figure 3:
To increase the number of GSCs available for analysis, the authors perform switch experiments in bam- ovaries. They again report a non-overlapping H3 distribution pattern, similar to wild type. However, the authors do not indicate the efficiency of the dual-switch system in this genetic background e.g. does nanos expression or cell cycle progression occur as expected? Moreover, how do the authors reconcile the fact that bam mutant GSCs undergo symmetric (and not asymmetric) divisions? Further validation of the switch system in this genetic background is required. At the very least, the authors should cells the number of cells within a single germarium that showed this pattern.

We agree with this reviewer that in this mutant background, there is no GSC/CB differentiation and no asymmetric cell division to give rise to two daughter cells with distinct cell fates. Therefore, we used this tumor model only to study old *versus* new histone incorporation pattern, but not their segregation pattern. This is because we found that the distribution of old and new histone H3 is similar in *bam* mutant GSC-like germ cells and *wild-type* GSCs at prophase to prometaphase, as shown in Figure 3. The segregation pattern is further studied using *wild-type* GSCs in Figure 4. The *nanos-Gal4* driver turns on a transgene expression in all germ cells in *bam* mutant ovary. To clarify this point, we have included a Supplemental figure panel to show this (see EV Figure 5A-B). The same criteria for wild-type GSCs (Figure 1) also applies to *bam* mutant GSC-like cells in all data analyses.

Fig 3F/G/H: It is difficult to see the overlap between the oligopaint signals and old or new H3 signals. The authors need to show a zoom and individual channels to understand better precisely

what was quantified. Also, the authors should comment on why the four oligopaint foci were of unequal size. Is this perhaps due to different levels of chromatin condensation, which might, in turn, condense or spread H3 signals?

To clarify this, we added zoom-in images for Fig.3F-H; we also added individual Z stack for the FISH signal in revised Figure 3.

4.Comments related to Figure 4:
Fig 4A-D: As with Figure 3, it is difficult to see the overlap between the oligopaint signals and old or new signals. The authors need to show a zoom and individual channels to understand what was quantified in each case. In the examples shown, the red or green H3 signal appears to be very diffuse and I do wonder how different levels of chromatin condensation/compaction might influence quantitation. Have the authors tried to address this in their quantitation method?

To clarify this, we added zoom-in images for Fig.3F-H; we also added individual Z stack for the FISH signal. In telophase cells, chromosomes start to decondense and we tried to reduce this influence by choosing a same area of Region Of Interest (ROI) around all four FISH dots to quantify old *versus* new histone signals. On the other hand, these four "dots" label the same gene region at duplicated maternal and paternal chromosomes. Even though their chromatin condensation/compaction may be different from other genomic regions, their relative condensation degree should not be drastically different, based on our current knowledge regarding the overall epigenome structure between sister chromatids and between homologous chromosomes.

Fig 4A: This image should show a GSC probed for dad in which all four dots are resolved. On a related note, was the two dot pattern ever observed in the bam mutant GSCs? If not, this might indicate potential cohesion defects in this genetic background.

Yes, the two-dot pattern was also observed in the *bam* mutant GSCs (see EV Figure 5G). The resolving of sister chromatids depends on the progression of mitosis: At early prophase, sister chromatids are bound by cohesin, which is later resolved at the chromosomal arms at prometaphase and finally at the centromeric region at metaphase to anaphase. We have shown this in a previous publication (Ranjan et al., 2019).

Fig 4B: Are these the 4 dots indicated by the arrows? The arrangement looks different. Perhaps number the 4 dots for clarity.

We changed this label with #1-4 to clarify.
Fig 4C/D: Given that the dad gene seems to localise closer to the poles than the bam gene, is it possible that this affects comparisons in H3 signals due to different levels of chromosome compaction? The authors should address whether chromatin compaction might affect the quantitation method. Moreover, with respect to resolution, by a very rough estimation, it is possible that each focus contains 100s or 1000s of nucleosomes. How can the authors be sure these H3 containing nucleosomes are associated with this specific gene? ChIP experiments might

not be possible in these tissues, but perhaps super-resolution imaging might help to improve the resolution of this experiment.

Indeed, we have strived to increase the spatial resolution in these experiments using STED, PALM and STORM. However, STED works the best with two channels at 568nm and 633nm and require super bright signals, which is incompatible with our need of at least three signals (old H3, new H3 and FISH signals) as well as the difficulty to turn on sufficient transgene expression in early-stage female germline, a conundrum well known in the *Drosophila* germ cell field. Both PALM and STORM are also technically challenging with all three channels to give out comparable blinking fluorescence. In addition, with these two superresolution methods, we cannot be 100% sure that all labeled histone molecules are excited. Therefore, a separation between old and new H3 could be due to excitation of a subset molecules of them. We would need more technical advancement and/or optimization to apply these methods to these *in vivo* studies.

On the other hand, Zeiss LSM 880/980 with Airyscan and GaAsp detectors was used for Airyscan superresolution mode with a 63x, Plan Apochromat (1.4 NA) oil objective, which can achieve a X-Y spatial resolution of ~ 150 nm. We have applied this method in a few previous publications (Ma et al., 2020; Ranjan et al., 2019; Wooten et al., 2020; Wooten et al., 2019), which show compatibility of this method with our system and the sufficient resolution to resolve sister chromatids in mitotic germ cells.

Finally, given that the measured differences in old H3 retention between dad and bam genes is not huge (59% compared to 48%), I would request that the authors carry out the same analysis for an additional gene predicted to be on/off in GSCs (ideally located on a different chromosome) and also for a neighbouring gene that might be expected to show a similar pattern.
Up to date, there is no single-cell RNA-seq that shows differential gene expression between GSC and CB at single-cell resolution. Therefore, we do not really have additional candidate to include in this assay. In fact, the *bgcn* gene was initially designed to be a "differentiation" gene just like *bam* gene. However, after we performed RNA FISH experiment, we found that it is expressed in both GSC and CB cells. On the other hand, we could add the two control genes, *ss* and *bgcn*, to the OligoPaint assay using WT GSCs at anaphase to telophase. We did not do that in the initial submission because very few GSC-like *bam* cells displayed the 2:0:2 ratio at these two gene loci, we focused on differentially expressed *dad* gene and *bam* gene in WT GSCs. However, if we need to add these, it will be a significant amount of work, given the extremely short anaphase and telophase in female GSC cell cycle. If we need to do this, we would need at least 6-month time for the revision, considering the current partial lab lockdown due to the pandemic situation.

General comment on the proposed model: The authors propose a model by which old and new H3 are differentially inherited at stem or differentiation genes. How do they envision these specific genes are recognised at the replication fork? Do the authors propose unidirectional fork movement as observed in males, but instead at specific chromosomal loci?
This is a great suggestion for the follow-up study, which will need technical advancement to examine fork movement using chromatin or DNA fibers isolated from female GSCs with probes recognizing specific genomic regions. We are currently developing these new methods but it will be beyond the scope of the current manuscript.

Referee #2:

The manuscript by Kahney et al. addresses how old versus new histones are partitioned during asymmetric division of Drosophila ovarian germline stem cells (GSCs) and their daughter cells. This is a fundamental question in cell and developmental biology. The Chen lab previously demonstrated in Drosophila male GSCs that the old histone H3 and H4 are segregated to the daughter GSCs but the new H3 and H4 enriched in gonioblasts. In this paper, the authors examined whether such an asymmetric pattern of histone segregation is conserved in female GSCs. There are three major findings in this paper:

First, the authors described the segregation of different histones in different types germ cells during early oogenesis. They showed that the old and new H3 chromosome domains are non-overlapping, that this non-overlapping pattern is most prominent during the GSC division, still very discernable during the cystoblast division, but are not obvious during cystocyte divisions. In addition, the authors reported that the old and new H4 molecules are also differentially segregated between GSCs and cystoblasts, but to a less extent than H3. However, H2A does not show any significant pattern of differential segregation during the divisions of GSCs, cystoblasts, and cystocytes. Moreover, the authors showed that, despite the asymmetric segregation of H3 with respect to individual chromosome domains during GSC and CB divisions, this asymmetry is overall not significant at the whole genome level.

Second, the authors showed that the non-overlapping segregation of old versus new H3 molecules in bam mutant germ cells is similar to that of wildtype GSCs. This discovery implicates that the bam mutant germ cells are more similar to GSCs than cystoblasts, which helps to clarify a long-standing uncertainty in the field.

Third, the authors creatively used the Oligopaint IF-FISH method to reveal that regions displaying differential distribution of old versus new H3 contain differentially expressed genes that function in cell fate regulation but not constitutively active or silent genes. This is an important proof-of-principle step towards deciphering the functional significance of differential histone segregation during cell division.

The discoveries reported in this paper are all fundamental to cell and developmental biology. They are based on quantitative analyses that are meticulously conducted and appropriately interpreted. These discoveries reveal the complex nature of asymmetric histone segregation with regard to both histone types and cell types that are previously unappreciated. The paper is overall well written and a pleasure to read. I recommend its publication without any major revision. Before the manuscript goes to press, I suggest a minor revision to address the following questions.

1. P5, para 2, line 5: "which can undergo ACD at their apical tips (Xie & Spradling, 2000)." The first report on ACD of female GSCs is Lin and Spradling in 1997 (Development, 124, 2463-2476) with a systematic description by Deng and Lin in 1997 (Dev. Biol. 189, 79-94).

We thank this reviewer and have added these critical references.

2. P6. Para1. Lines 6-10: The first description of asymmetric CB division which would create oocyte versus nurse cell fate was by Lin and Spradling in 1995 (Developmental Genetics 16, 6-12.), and the first demonstration that asymmetric spectrosome inheritance may play a role in such a cell fate determination was by Deng and Lin in 1997 (Dev. Biol. 189, 79-94).

We thank this reviewer and have added these critical references.

3. P11, para 2: The division of GSCs, CBs, and CCs are all equally asymmetric, at least with regard to spectrosome/fusome segregation, subsequent microtubule network formation, and eventual oocyte determination, with the initial CB division presumably pre-determines the oocyte fate (e.g., Lin and Spradling, 1995). Hence, the statement "it is possible that breaking the symmetry in preparation for cellular differentiation in the female germline lineage is accomplished by two steps, GSC division and CB division" is unlikely to be accurate because it is only reasonable to suggest that either all divisions break the symmetry (if the authors does not define "breaking symmetry" as a single step) or only CB division is the breaking step (if the authors define "breaking symmetry" as a single step) for oocyte determination. In either scenario, it would not be a two-step process involving just GSC and CB division.

Due to limited space, these rather unclear statements have been deleted in this revision.

4. The titles of the last two sections are too similar. The authors may want to rephrase the titles to emphasize more on the unique aspects of each of the two sections.

We thank this reviewer and have revised them accordingly.

5. The last two sections are lengthy and somewhat repetitive. If the authors can make these two sections more succinct, that will be helpful to readers.

We thank this reviewer and have revised this part accordingly.

Referee #3:

This report follows up on previous work from the Chen lab showing that old histones preferentially segregate into the stem cell during the asymmetric divisions of Drosophila male germline stem cells by examining whether this also occurs in the female germ line. They observe a non-overlapping distribution of old and new Histone H3 in the germline stem cell and its daughter, the cystoblast, whereas other Histones show a more uniform distribution. However, the old and new histones show no significant preferential segregation into either daughter during these divisions. The authors then hypothesize that the histones may be asymmetrically inherited on specific loci that control stemness versus differentiation and use in situ hybridisations to suggest that this might be the case at the dad locus.

While the experiments appear to have been well done, the manuscript falls short of proving its main conclusion that old Histone H3 preferentially segregates on the copies of e.g. the dad gene inherited by the stem cell daughter. The assay using Oligopaint FISH lacks the resolution needed to assign the gene to a particular histone territory and the data are analysed in a way that amplifies small differences, so that an allele that has 51% of old H3 versus 49% of new H3 is scored the same way as an allele with 100% to 0%.

The results are not striking despite this analysis method and this is further compounded by the fact that the assay cannot distinguish between the two copies of the maternal chromosome after replication and the two copies of the paternal chromosome.

The designed Oligopaint FISH probes cover the entire ~50-kb genomic region at the designated gene and should provide the resolution to assign the gene to a particular chromatin region.

On the other hand, we are striving to come up with a good assay for these data. Some technical and biological complications include (1) only a subset of histones are labeled using the transgene, all the endogenous histones are unlabeled and will not be visualized. Currently it is not possible to tag all endogenous histone genes, given that there are 24 copies of them and it is unclear whether their expression has any cell type- and stage-specificities. (2) The mitotic chromosomes are at the most condensed state during cell cycle, which provides an opportunity to visualize old *versus* new histone-enriched domains. However, this also serves as a challenge to detect separation between old and new histone signals. Currently it is technically unfeasible to visualize old versus new histone distribution on interphase chromosomes given their mostly decondensed state.

Even though we agree with the reviewer that the difference could be subtle due to the above reasons, the separation between old and new H3 in germline stem cells can be detected and such a separation is statistically significant compared to old and new H3 in late-stage germ cells (i.e. cell stage specificity) and to old and new H2A in germline stem cells (i.e. molecular specificity).

In the revision, we could add the two control genes, *ss* and *bgcn*, to the OligoPaint assay using WT GSCs at anaphase to telophase, in order to compare the results using *dad* and *bam* genes. At anaphase and telophase, sister chromatids for both maternal and paternal chromosomes are already separable and pulled to the two poles of GSCs.

However, if we need to add these, it will be a significant amount of work, given the extremely short anaphase and telophase in female GSC cell cycle. If we need to do this, we would need at least 6-month time for the revision, considering the current partial lab lockdown due to the pandemic situation.

The authors try various tricks to make these effects seem more significant, but in my opinion they are trying too hard:

"However, the normalization scheme could potentially create a situation in which one dot has both the highest GFP and the highest mCherry signal, leading to a 2:1:1 pattern (see Materials and Methods), which is likely due to the high condensation of chromosomes in mitotic cells. If we consider both the 2:0:2 and the 2:1:1 pattern, approximately 71% of dad and 71% of bam FISH signals have a preferential association with old versus new histones. For the ~30% of signals where a preferential association was not detected with either old or new histones, it is possible that fewer labeled histones were incorporated at that genomic region, or that the chromatin was folded and condensed in such a manner that immunostaining was unable to detect it."

For example, the last sentence attempts to dismiss the nuclei that show equal segregation by claiming that they show less total signal for some other reason, but they present no evidence to support this view. They also fail to apply the same critical thinking to the nuclei that they score as showing asymmetric inheritance, which raises a concern about a bias in the analysis. I therefore think that these results need to be confirmed by another method, such as super-resolution imaging, for the conclusion to be compelling. For this reason, I think that this manuscript is too speculative for publication.

In addition to the points discussed above, we have strived to increase the spatial resolution in these experiments using STED, PALM and STORM. However, STED works the best with two channels at 568nm and 633nm and require super bright signals, which is incompatible with our need of at least three signals (old H3, new H3 and FISH signals) as well as the difficulty to turn on sufficient transgene expression in early-stage female germline, a conundrum well known in the *Drosophila* germ cell field. Both PALM and STORM are also technically challenging with all three channels to give out comparable blinking fluorescence. In addition, with these two superresolution methods, we cannot be 100% sure that all labeled histone molecules are excited. Therefore, a separation between old and new H3 could be due to excitation of a subset molecules of them. We would need more technical advancement and/or optimization to apply these methods to these *in vivo* studies.

On the other hand, Zeiss LSM 880/980 with Airyscan and GaAsp detectors was used for Airyscan superresolution mode with a 63x, Plan Apochromat (1.4 NA) oil objective, which can achieve a X-Y spatial resolution of ~ 150 nm. We have applied this method in a few previous publications (Ma et al., 2020; Ranjan et al., 2019; Wooten et al., 2020; Wooten et al., 2019), which show compatibility of this method with our system and the sufficient resolution to resolve sister chromatids in mitotic germ cells.

Other points:

1) The evidence for the uneven distribution of old and new Histone H3 in post S-phase nuclei is strong, but the authors consider only one possible explanation for this hypothesis, which is that the old Histone is being preferentially inherited by one strand of the DNA after replication. In my opinion, a more likely explanation is that the non-overlapping distributions of new and old Histone H3 corresponds to when in S-phase the DNA is replicated, as the new Histone gradually increases over time after the Flip from the green to the red version. This would suggest that the chromosomal territories that are predominantly labelled with new H3 are late replicating heterochromatic regions.

We thank this reviewer for the suggestion. However, based on our preliminary data, the old H3 signal tends to be enriched at the poles in telophase GSCs, which could be due to old histone retention at heterochromatin. Based on the heat shock regime and the cell cycle length of female GSCs (Figure 1B), when the GSCs undergo the S phase after heat shock, the old H3-coding sequence had been flipped out, the majority of the labeled new H3 has the other fluorescence label that are incorporated into the duplicating genome throughout the S phase.

2) "The cellular specificity of the non-overlapping old versus new histone H3 patterns recapitulates what has been previously reported in Drosophila male GSCs, where the global asymmetric inheritance of old versus new H3 is specifically found in asymmetrically dividing GSCs but not in symmetrically dividing spermatogonial cells (Tran et al., 2012)."

While this statement is technically correct, it is very misleading. In the male germline stem cell, all of the new and old H3 segregate from each other into different daughters at division, which is not the case in the female germ line. It would be more pertinent to point out that the answer to the question "whether differential histone inheritance is a conserved feature of stem cells and/or asymmetrically dividing cells or not" is a clear no.

We have revised this part to point out the differences between these two systems. However, we want to point out that the spatial separation with old *versus* new H3 but not with old *versus* new H2A in prophase GSCs is similar between these two systems; and the disappearance of the separation during germline differentiation is also similar between these two systems. We have never claimed that the global asymmetry is conserved. In fact, a very important point of this manuscript that the old *versus* new H3 asymmetry could be global as in the *Drosophila* male GSCs (Tran et al., 2012) and *Drosophila* intestinal stem cells (bioRxiv, doi: https://doi.org/10.1101/2020.08.15.252403), or local as in the *Drosophila* female GSCs (shown in this manuscript) and in Wnt3a-induced mouse embryonic stem cells (Ma et al., 2020). Since different stem cell systems have distinct cellular differentiation programs and different changes in gene expression profile, we hypothesize that these differences are related to the degree of histone asymmetry displayed during asymmetric stem cell division. This point has been discussed in (Ma et al., 2020). As what has been pointed out by Reviewer#2, these new findings “reveal the complex nature of asymmetric histone segregation with regard to both histone types and cell types that are previously unappreciated”.

3) "Interestingly, a subset of GSC and CB divisions results in two daughters with biased old versus new H3 and H4 inheritance, reflected by a wide distribution of H3 and H4 compared to H2A, which has a more clustered distribution (Fig 2E, 2J)".

Since this subset turned out not to be significant on further analysis, why are they mentioned at all.

We have left out this discussion.

4) "Furthermore, the similarity of old versus new histone distribution patterns among WT GSCs, WT CBs, and bam mutant germ cells (data re-plotted in Fig EV3D for direct comparison) shed light on a long-held debate in the field whether bam mutant germ cells resemble more like GSCs or CB".

This analysis cannot shed light on whether bam mutant germ cells are more like GSCs or CBs, because GSCs and CBs are indistinguishable in their distributions of new and old histones.

We have left out this discussion.

References:

Lin, S., Yuan, Z.F., Han, Y., Marchione, D.M., and Garcia, B.A. (2016). Preferential Phosphorylation on Old Histones during Early Mitosis in Human Cells. J Biol Chem *291*, 15342-15357.

Ma, B., Trieu, T.J., Cheng, J., Zhou, S., Tang, Q., Xie, J., Liu, J.L., Zhao, K., Habib, S.J., and Chen, X. (2020). Differential Histone Distribution Patterns in Induced Asymmetrically Dividing Mouse Embryonic Stem Cells. Cell Rep *32*, 108003.

Ranjan, R., Snedeker, J., and Chen, X. (2019). Asymmetric Centromeres Differentially Coordinate with Mitotic Machinery to Ensure Biased Sister Chromatid Segregation in Germline Stem Cells. Cell Stem Cell *25*, 666-681 e665.

Tran, V., Lim, C., Xie, J., and Chen, X. (2012). Asymmetric division of Drosophila male germline stem cell shows asymmetric histone distribution. Science *338*, 679-682.

Wooten, M., Li, Y., Snedeker, J., Nizami, Z.F., Gall, J.G., and Chen, X. (2020). Superresolution imaging of chromatin fibers to visualize epigenetic information on replicative DNA. Nat Protoc *15*, 1188-1208.

Wooten, M., Snedeker, J., Nizami, Z.F., Yang, X., Ranjan, R., Urban, E., Kim, J.M., Gall, J., Xiao, J., and Chen, X. (2019). Asymmetric histone inheritance via strand-specific incorporation and biased replication fork movement. Nat Struct Mol Biol *26*, 732-743.

Xie, J., Wooten, M., Tran, V., Chen, B.C., Pozmanter, C., Simbolon, C., Betzig, E., and Chen, X. (2015). Histone H3 Threonine Phosphorylation Regulates Asymmetric Histone Inheritance in the Drosophila Male Germline. Cell *163*, 920-933.

1st Revision - Editorial Decision 8th Mar 2021

Dear Dr. Chen,

Thank you for the submission of your revised manuscript and the friendly video chat to clarify the revisions.

To summarize, as we agreed, you will try to add more samples to figures 3 and 4. At the same time you will explain the quantitations better and may be show more examples. All comments by referee 1 must be addressed.

A few other editorial changes will also be required:

- Please move all methods to the main manuscript file. The supplemental file can be deleted.

- Please correct the reference format to the EMBO reports (Harvard) style that can be found in EndNote. Not more than 10 authors should be listed before "et al".

- The FUNDING INFO in our online manuscript handling system and the manuscript itself don't match, please correct.

- Fig 1 panels are not called out alphabetically, Fig 2K callout is missing,
Fig EV1 panel callouts are missing, Fig EV3 panel callouts are missing. There is a callout to EV3D which doesn't exist. Fig EV4A,B+D panels are missing. Please correct.

- For the DATASETs, the file name is missing within the files. Dataset EV3 is in two files, this must be combined. Please add the titles and legends of every Dataset to the first tab of the same excel file, so that all information is on one page.

- Please move the figure legends to after the references.

I attach to this email a related manuscript file with comments by our data editors. Please address all comments in the final manuscript.

EMBO press papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is exactly 550 pixels wide and 200-600 pixels high (the height is variable). You can either show a model or key data in the synopsis image. Please note that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

I look forward to seeing a final manuscript when it is ready.

Kind regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

In this revised version, the authors have adequately addressed my concerns related to potential 'dilution' effects due the timing /induction of the GFP/mCherry switch, now shown in new EV Figures 1 and 5 and through additional clarifications in the text.

In my opinion, the inclusion of ss and bgcn probes in Figure 4 would significantly strengthen this manuscript. Without these additional experiments, perhaps the addition of a few more labels to Figures 3 and 4 would clarify which signal was quantified in each case. This would improve the overall interpretation of this set of experiments. For example:

-Figure 3F-H has been improved by the addition of the zoomed-in panels. It would also be helpful if the authors indicated in each representative case whether a focus is scored positive or negative for 'red' or 'green' signal. For example, of the 4 foci highlighted for the dad probe in 3F, which 2 are scored positive for red and which 2 are scored positive for green? The same applies for Figures 3G, 3H and 4C, 4D.

-I am still a bit confused by Figure 4A. Why are only 2 dad foci shown in this prophase GSC? Were nuclei with 2 or 4 foci included in the analysis? What is meant by side 1 and side 2 - are these the signals of the resolved sisters? Perhaps it would be best to show a representative cell in which 4 dad foci are clearly visible?

Finally, in Figure 2F-I, the authors added an inset zoom of the anti-spectrin staining, but I am still not sure what it is indicating? It is hard to tell whether the signal is present or absent in each image and this should be further clarified.

Referee #3:

The authors have dealt with all of my minor comments. However, I remain unconvinced that they have strong evidence that there is asymmetric segregation of old versus new Histone H3 on specific loci such as dad. They did not address the issue that I raised about the quantification of these data and and are not currently able to visualise specific loci with sufficient resolution to resolve whether they fall in a region marked by old or new histones. I am therefore remain sceptical about the main conclusion of the manuscript and cannot see anything that they could reasonably do to address this issue.

Further comments by referee 1:

Yes, I would support publication of this study if the authors explained and labelled their quantitations better. Referee 3's point that that 'an allele that has 51% of old H3 versus 49% of new H3 is scored the same way as an allele with 100% to 0%' is a valid one and perhaps they could address this also.

I do think the histone segregation data in the first two figures is robust and very interesting.

Point-by-point responses to reviewer's questions and requests:

Referee #1:

1. About quantification: "an allele that has 51% of old H3 versus 49% of new H3 is scored the same way as an allele with 100% to 0%" "how many loci in total you counted and how prominent the asymmetric distribution is."

   We now added the old H3% versus new H3% to each image panel with FISH signals in Figure 3 and Figure 4, as well as all results in the Dataset_EV3 and Dataset_EV4, along with the ratio calls, such as 3:0:1, 2:0:2, etc. This way the readers can clearly see the quantification results and how these results are converted to more straightforward ratio calls. Even though this particular case this Reviewer brought up is possible, the actual data rarely fall in this extreme category.

2. "In my opinion, the inclusion of ss and bgcn probes in Figure 4 would significantly strengthen this manuscript. Without these additional experiments, perhaps the addition of a few more labels to Figures 3 and 4 would clarify which signal was quantified in each case. This would improve the overall interpretation of this set of experiments."

   We now improved presentation on Fig.3F-H and 4A-B by adding the information of old H3% versus new H3% next to the different ratio calls. We also added the old H3% versus new H3% to the Dataset_EV3 and Dataset_EV4, which include all data presented in Figure 3 and Figure 4.

   For the data in Figure 4 we now include a gallery for all telophase cells for *dad* FISH and *bam* FISH, along with old and new H3 signals, respectively, in Fig.EV6.

3. "I am still a bit confused by Figure 4A. Why are only 2 dad foci shown in this prophase GSC? Were nuclei with 2 or 4 foci included in the analysis? What is meant by side 1 and side 2 - are these the signals of the resolved sisters? Perhaps it would be best to show a representative cell in which 4 dad foci are clearly visible?"

   We now replaced Figure 4A and used exclusively anaphase or telophase images in Figure 4. We also added more examples of anaphase or telophase images in Figure 4.

4. "Finally, in Figure 2F-I, the authors added an inset zoom of the anti-spectrin staining, but I am still not sure what it is indicating? It is hard to tell whether the signal is present or absent in each image and this should be further clarified."

   In these figures, the spectrosome structure was shown by anti-α-spectrin staining, which is a round structure and is better illustrated in the insets. This signal is less obvious just because the other signal in the same channel shows H3S10 phosphorylation using anti-H3S10P, which is very bright in mitotic cells.

2nd Revision - Editorial Decision 16th Apr 2021

Dr. Xin Chen
Johns Hopkins University
Biology Department
Levi Hall 137
3400 North Charles Street
Baltimore, MD 21218-2685
United States

Dear Dr. Chen,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: emboreports@embo.org]. If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

Should you be planning a Press Release on your article, please get in contact with emboreports@wiley.com as early as possible, in order to coordinate publication and release dates.

Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Best regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

****

Referee #1:

In this revised version of the manuscript, the addition of % scores to the representative images in Figures 3 and 4 have greatly improved the presentation of findings and have clarified the interpretation of these results. The inclusion of additional examples in panels 3F-H and 4A-B is also very helpful. It is also reassuring to see that the data did not fall into the rare category of 51% old: 49% new. In the telophase images shown in Figure 4, the designation of GSC side or CB foci and how each comprised two measurements is now clearer also.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Xin Chen
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2020-51530V1

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Samples were collected until the mean of the data was no longer changing.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Sample size estimates were selected based on field standards.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	All female flies of the correct age with cells at the correct stage were included.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	To minimize subjective bias in group allocation, flies from a single vial were evenly spread across treatments.
For animal studies, include a statement about randomization even if no randomization was used.	No randomization was used.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	A strict quantification protocol was established and followed to minimize subjectivity when analyzing results.
4.b. For animal studies, include a statement about blinding even if no blinding was done	No blinding was done.
5. For every figure, are statistical tests justified as appropriate?	Yes.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes, diagnostic plots of the residuals were created to assess the models assumptions using the qqnorm and qqline commands in R Studio.
Is there an estimate of variation within each group of data?	Yes, all data is marked with 95% confidence intervals.
Is the variance similar between the groups that are being statistically compared?	Yes, all data being statistically compared uses a pooled standard deviation.

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumo

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jij.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	The primary antibodies used were mouse anti-alpha-Spectrin (1:50, DSHB, Cat# 3A9), mouse anti-hu-li tai shao (1:50, DSHB, Cat# 1B1), mouse anti-Armadillo (1:100, DSHB, Cat# N27A1), mouse anti-H3S10ph (1:5000, Abcam, Cat# ab14955), rabbit anti-GFP (1:200, Abcam, Cat# ab290), rabbit anti-GFP (1:400, Invitrogen, Cat# A-11122), Chicken anti-mCherry (1:1000, Novus Biologicals, Cat#NBP2-25158). Secondary antibodies were the Alexa Fluor-conjugated series used at 1:1000 (goat anti-mouse 405, goat anti-rabbit 488, goat anti-chicken 568; Molecular Probes).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	This study uses Drosophila melanogaster females within 5 days post-eclosion. Fly stocks were raised using standard Bloomington medium at 25°C. The following fly stocks were used: Heatshock-flippase on the X chromosome (Bloomington Stock Center BL-26902), GreenEye-nanos-Gal4 on the 2nd chromosome [Bloomington Stock Center BL-32179, from Dr. Daniela Drummond-Barbosa, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland, USA, (Holtzman, Miller et al., 2010)], UASp-FRT-H2A-eGFP-PolyA-FRT-H2A-mCherry on the 3rd chromosome, UASp-FRT-H2A-eGFP-PolyA-FRT-H2A-mCherry on the 2nd chromosome, UASp-FRT-H3-eGFP-PolyA-FRT-H3-mCherry on the 2nd chromosome, and UASp-FRT-H4-eGFP-PolyA-FRT-H4-mCherry (2nd chromosome) (Wooten, Snedeker et al., 2019), UASp-FRT-H3-mCherry-PolyA-FRT-H3-eGFP on the 3rd chromosome (Ranjan, Snedeker et al., 2019), nanos-Gal4 (Van Doren, Williamson et al., 1998) and bamΔ86 (Bopp, Horabin et al., 1993) combined on the 3rd chromosome (from Dr. Mark Van Doren, Johns Hopkins University, Baltimore, Maryland, USA), bam1 on the 3rd chromosome (McKearin & Spradling, 1990). Transgenic flies with the following transgenes were newly generated in studies reported here: UASp-FRT-H3.3-eGFP-PolyA-FRT-H3.3-mCherry on the 2nd chromosome.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Confirmed

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	All data tables are provided as part of the Supplmental Information.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No