JCB

Asymmetric assembly of centromeres epigenetically regulates stem cell fate

Anna Dattoli, Ben Carty, Antje Kochendoerfer, Conall Morgan, Annie Walshe, and Elaine Dunleavy

Corresponding Author(s): Elaine Dunleavy, National University of Ireland Galway

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November 7, 2019

Re: JCB manuscript #201910084

Dr. Elaine Dunleavy
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Dear Dr. Dunleavy,

Thank you for submitting your manuscript entitled "Asymmetric assembly of centromeres epigenetically regulates stem cell fate". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

You will see that the reviewers found your analyses of CID deposition in the female Drosophila germline interesting and of quality. The referees provided what we feel are constructive and valid suggestions to strengthen your conclusions, deepen the discussion of the results, in particular in light of recent published work, and clarify the results and manuscript for a broad audience. Efforts should be dedicated in revision to address these points in full, to the best of your ability. In response to Rev#2 point #3, we recommend that you adjust the conclusion to take into account the depletion did not work or provide evidence that the depletion was effective. Please do not hesitate to contact us if you have any questions or anticipate any issue addressing these comments. We would be happy to discuss the revisions as needed.

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

GENERAL GUIDELINES:

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

Figures: Articles may have up to 10 main text figures. Figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, http://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

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

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

The typical timeframe for revisions is three months; if submitted within this timeframe, novelty will not be reassessed at the final decision. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

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

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

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

Sincerely,

Arshad Desai, PhD
Editor, Journal of Cell Biology

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

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

The histone variant CID/CENP-A provides the foundation for centromere function. Prior work has explored its propagation extensively in cell culture, but it is still unclear how its deposition is altered in the context of cells in an organism. In this paper, Dattoli et al. explore CID deposition in the female germline divisions in Drosophila. They find that CID is incorporated during G2 / early mitosis based on an increase in CID fluorescence. They additional show that this incorporation is promoted by Cyclin A and prevented by HASPIN kinase. Finally, they provide data to support an asymmetry to CID levels between the two sister kinetochores, and show that perturbations that disrupt CID asymmetry also lead to changes in stem cell fate (self renewal, etc).

Overall, there is a lot of interesting data in this paper, I am supportive of this paper being published in the JCB. However, there are several changes that would improve this paper and that I would recommend prior to publication. Many of these could likely be addressed through changes to the text, including commenting specifically on aspects of the data, using caution with interpretations, or additionally mentioning alternate models.

1. The asymmetry of CID on the sister chromatids in a stem cell division has potentially substantial consequences. Recent work from the Chen lab working in the male germline in Drosophila has reported a similar asymmetry, and has argued that this is the basis for the asymmetric chromosome segregation observed in some stem cell divisions. This model is appealing, but I found the imaging

and quantitation in the Chen lab paper (Ranjan et al., Cell Stem Cell 2019) to hard to interpret and not completely convincing. Thus, this paper from Dattoli et al. provides an important addition to the field be providing robust data that is more clear. At the same time, the results observed here are not completely consistent with this model in that the asymmetry of CID intensity seems quite modest to me. Here, the authors find a ratio of 1.2 for CID intensity in the GSC vs. CB side. In contrast, Ranjan et al. report an average ratio of about 1.7, with some cells showing a 2 to 2.5 fold difference. It is much harder to imagine that a 1.2 fold difference in CID levels would result in a functional difference between the two sister kinetochores. In this case, the work from Dattoli could be interpreted as arguing that CID asymmetry is not a substantial source of differences in these divisions. I would appreciate it if the authors could comment on these ratios, their interpretations, and differences from the Chen lab paper.

2. Related to the point above, it is quite striking that CID or Cal1 overexpression and Haspin knockdown results in an increased number of GSCs. This could be interpreted as disrupting an asymmetry to chromosome segregation, with a subsequent effect on cell fate. However, given the nature of these perturbations, it is important to use caution in interpreting these behaviors. For example, it is possible that these changes affect chromosome structure at non-centromere loci, altering the expression of key gene. Alternately, these perturbations may alter nucleolar function. Indeed, recent work from the Yamashita lab has pointed to an important role for rDNA and the nucleoli in key stem cell behaviors and possibly chromosome distribution. It would be helpful if the authors comment highlight these different possibilities, but also use caution in some of their interpretations.

3. The effect of Haspin on CID deposition is interesting, and to me unexpected, although H3T3 has been implicated in aspects of asymmetric cell division behaviors. However, it is not clear to me what the basis for this is. For example, Haspin could act through H3T3 phosphorylation, or could directly target Cal1 or another factor. The authors even use H3T3P as a marker, but don't correlate this with CID desposition or centromere asymmetries. Is it possible to monitor the relative timing and co-localization of the H3T3 mark and asymmetry with that of CID using their existing data? I would also appreciate it if the authors could speculate about the potential inhibitory role of Haspin.

4. There are some sections of the text that would be much more accessible if the authors were to streamline these substantially. For example, the first section on the cell cycle distribution of centromeres provides a nice demonstration of their ability to follow these events, but does not provide extensive impactful information that is critical to the conclusions in the paper. There are also sections where it is important for the authors to test the effect in their system, but where the result is not particularly surprising, such as the role of Cal1 in depositing CID.

5. There are also some wording choices that seem a bit odd. The repeated use of "Following" to start a sentence seemed awkward to me, for example.

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

This study by Dattoli et al., explores the assembly of the centromere-specific histone CENP-A (CID in Drosophila) in the stem cells of the Drosophila female germline. They report that CID assembly occurs between G2 and prophase, promoted by Cyclin A and inhibited by Cyclin B and Haspin. In the germarium of the Drosophila Ovary, germline stems cells (GSCs) divide into two daughters, one which maintains stemness (GSC) and the other which will differentiate into a cytoblast (CB).

Interestingly, they find that cell fate is connected to asymmetric distribution of CID and CENP-C between the different sister chromatids. They provide support for a model that this might be linked to creating kinetochores of different strength, the stronger kinetochore residing in the GSC cells and bias segregation. Overexpression of CENP-A alone or with its chaperone CAL1 or knockdown of Haspin eliminates asymmetry and drives stem cell renewal at the expense of cell differentiation.

The link between asymmetric CID assembly and cell fate is an important and exciting finding that is well supported by the presented data. While this is the key message of the paper, other conclusions such as the CID loading time or its role in later stages are less clear and need further experimental and analytical support. The paper is in parts difficult to read and the writing style could be improved where indicated.

Major points:

1) As a major point, I don't find that there is strong data supporting that CID assembly already starts in G2. The figures and the text refer to G2/prophase, not distinguishing them.
On page 6 the authors themselves state that centromere assembly likely initiates during G2. Where is the data supporting this statement? Without providing further support, this statement later turns into the conclusion: "Our results confirm that similar to GSCs also neural stem cells assemble centromeres between G2 and prophase."

To me it is still possible that CID assembly starts in mitosis with prophase. The authors should be able to distinguish CID loading in G2 vs prophase by using chromosome condensation, nuclear envelope breakdown and H3S10p as markers.

Also, there is a noticeable increase in loading of CID from prophase to metaphase. The authors ignore this because it is not significant. However, at face value it looks not much different then increase between S phase and G2/prophase. Increasing the small N over 12 or 18 might easily give this result significance and would then change the conclusion. In other words loading could actually be happening from prophase to metaphase.

2) The statement: CYCB depleted germaria seem to have more
cells compared to the control (Fig. 2M-N')" is quite vague. This should be quantified.

3) The authors use the Bam-Gal4 driver to knock down CAL1 and CID at later stages of egg development. The don't observe any effect on cell division but they also state that CID levels are not particularly diminished compared to the control.

Isn't the most straightforward interpretation of this, that the knockdown simply didn't work?

Instead the authors continue to conclude: "These results indicate that CID and CENP-C are already assembled at centromeres at this stage and CAL1 function is dispensable at least for the cell division occurring after the 8-cell stage."
This is a bold statement, that is currently not backed up by the data. Without proper quantification of CID levels (microscopy or Westerns?), this and the subsequent conclusion in the discussion should be revised.

Minor points:

Abstract

The first phrase in the abstract make for difficult reading. Better: "Centromeres are epigenetically defined by CENP-A-containing chromatin and essential for genome integrity."

I find this statement and the use of the word "while" strange : ."..down or CENP-A over-expression drives stem cell self-renewal, while the CENP-A assembly factor CAL1 is crucial for cell division". First, the paper shows that CAL1 o/e together with CENP-A also drives stem cell-renewal, and second is this to say that CENP-A is not crucial for cell division?

Introduction:
Reference 25 is cited as BioRxiv but has since been published in Developmental Cell as is also referenced in this paper as 64. This reference should be updated.

The authors often speak of centromere assembly, when they actually mean CID (or CENP-A) assembly. This is confusing, as the centromere encompasses more than just CENP-A. Only later in the paper, when the binding of CENP-C and CAL1 also has been assessed does it make sense to use this more general term.

Again, the use of the word "while" as a conjunction doesn't make sense here and is confusing, when comparing Drosophila and human:
"In flies, CID (the homologue of CENP-A) deposition requires activation of the anaphase promoting complex/cyclosome (APC/C) and degradation of CYCA 21, while centromere
assembly is antagonised by Cdk1 activity and promoted by the kinase Plk1 in humans 29-31." - It suggests a contrast, even though CDK activity (and Cyclin B) also plays a role in antagonising CENP-A assembly in flies (also shown in this paper).

Page 4: typo: In Drosophila, CID binds to CAL1 (fly functional homologue of HJURP - missing")"

The connection between Cyclin B and Haspin should be introduced a little bit more detail already in the introduction with appropriate references (here it would be better to cite the original papers rather than the review (ref 54). Strangely there is no mention of CDK1, although Cyclin B is surely not acting alone.

Results:
In this study spectrosome shape and positon plays an important role in distinguishing GSCs and CBs. It would be helpful to shortly mention here how it is recognized (antibody 1B1) and expand here or later what position goes with what cell type (the shape is explained a little later in the paper).

Typo: Page 5, top: To achieve our aim...

Page 7, top: The authors state: "This is different from CYCB localization pattern..." - but fail to explain in what way it is different.

Page 7, top: Again, I would recommend to exchange "centromere assembly" with "CID assembly" because this is what is assayed.

Page 7, top: Replace "Following" with "Next,..." here and elsewhere in the paper.

Not clear, why the authors decided to examine endoreplication as the next thing. Please explain.

Page 8, bottom: For better understanding Figure 3 it would be helpful to mention in the text the correlation with the spectrosome, position. If I'm not mistaken this would be:... Following (better: Next), we measured the total amount of CID present on one set of chromosomes (spectrosome proximal) versus the other (spectrosome distal).

Page 9: The paper uses a paragraphs and Fig 4 to show that CAL1 is required for cell proliferation an dCID and CENP-C recruitment. Is that surprising? I would expect that any one of these centromere proteins are important for proliferation.

Page 9, middle: What is "enucleated"?

Page 9, middle: The authors state: "To confirm that the microtubules enucleated were captured by centromeres, we also performed a staining for both tubulin and CID (Fig. Fig. 3J-K').
-but the result is not described. Is it confirmed?

Page 10, top: Introduce the meaning of "agametic"

Page 11: "We first quantified the number of round spectrosomes (Fig. 5A-D and M)..."
- And that is indicative of what?

Page 12, middle:
I do not understand this statement: "In order to investigate whether asymmetric inheritance of CID between GSC and CB has a role in the stem cell niche disruption (Fig. 5)." - Isn't the asymmetric inheritance the normal situation? Why "disruption"?

On page 12: With the given order of the paragraphs, I get the impression the story is told backwards. After increasing the CID levels (by overexpression alone or with CAL1, or Haspin knockdown), wouldn't it first make sense to analyse CID levels (symmetric vs. asymmetric) before looking at the effect on cell fate?

End of Results, just before Discussion: The last conclusion should be rephrased for better understanding (assuming the statement is correct, see Major comment 3).

Discussion:
Page 15: I find the link to the Swartz et al. paper a little far-fetched. I don't see any particular logical connection between small differences in CENP-A levels determining cell fate, to small incremental loading of CENP-A which amounts to larger amounts of CENP-A over time.

In turn, I'm really surprised that the work of the Lampson lab is not cited in this paper, which provides support to the centromere drive hypothesis and similar to this study concludes that kinetochore strength has an impact on chromosome segregation and cell fate.

Methods:
EdU: It should give more information and say: Ovaries from young female flies were dissected...

Figure 1: The red writing in G and H is hard to read. Also, the white EdU and green CID are hard to distinguish.

Point-by-point response to reviewers' comments on JCB manuscript #201910084:

Response to Reviewer #1:

1. The asymmetry of CID on the sister chromatids in a stem cell division has potentially substantial consequences. Recent work from the Chen lab working in the male germline in Drosophila has reported a similar asymmetry, and has argued that this is the basis for the asymmetric chromosome segregation observed in some stem cell divisions. This model is appealing, but I found the imaging and quantitation in the Chen lab paper (Ranjan et al., Cell Stem Cell 2019) to hard to interpret and not completely convincing. Thus, this paper from Dattoli et al. provides an important addition to the field be providing robust data that is more clear. At the same time, the results observed here are not completely consistent with this model in that the asymmetry of CID intensity seems quite modest to me. Here, the authors find a ratio of 1.2 for CID intensity in the GSC vs. CB side. In contrast, Ranjan et al. report an average ratio of about 1.7, with some cells showing a 2 to 2.5 fold difference. It is much harder to imagine that a 1.2 fold difference in CID levels would result in a functional difference between the two sister kinetochores. In this case, the work from Dattoli could be interpreted as arguing that CID asymmetry is not a substantial source of differences in these divisions. I would appreciate it if the authors could comment on these ratios, their interpretations, and differences from the Chen lab paper.

We are in agreement with Reviewer 1 that there are some similarities, and some differences, between our findings and those which have been recently published by Ranjan et al. 2019. We believe the following factors might contribute to any differences and now refer to such possibilities in the 'Discussion'.

(i) One straightforward explanation is that male and female GSCs show different properties. Indeed, Ranjan et al. identify different categories of asymmetry: medium (1.2-1.4 fold difference) and strong (>1.4 fold). In males the asymmetry is shifted towards the second category (~60%), while it is possible that in females this asymmetry is shifted towards the first one.

(ii) Another difference is that the quantification method used to measure centromere intensity in our study was different to that used by Ranjan et al. In our study, we used an automated approach in image J/Fiji; after background subtraction of the entire image and adjustment of threshold with the default algorithm threshold, centromeres were selected automatically using the 'analyze particle' function. In contrast, Ranjan et al. manually drew the region of interest around both the fluorescent signal and the background. Furthermore, it is not clear what unit Ranjan et al. used to measure the fluorescence, i.e. the Gray Value or the Integrated Density, as these terms are used interchangeably in the description of their method. This is important as the gray value (=Mean Gray Value) indicates the average value of fluorescence for a specific region of interest, while the Integrated Density (MGV*area of the region of interest) takes into account also the size of the region of interest. This is crucial for quantitation at particular cell cycle stages when centromeres are highly clustered or when it is not possible to distinguish single centromere foci due to DNA condensation. We specify in our methods which value we reported for each experiment. Lastly, in order to include all centromere signals within the nucleus, we projected the entire nucleus with the use of different markers, i.e. DAPI, H2Av, VASA (which is cytoplasmic, but leaves the nucleus empty and clear to see) and then we measured the fluorescence of each centromere present in the nucleus. Differently, Ranjan et al. sum the "Gray Values" from each Z-stack of the region of interest (drawn manually) from its first appearance until the signal disappears.

(iii) In addition to measuring the amount of centromere/kinetochore protein per nucleus, Ranjan et al. also show quantifications regarding single centromere pairs. Yet, from the images presented in their study, it is difficult to distinguish single centromere foci. Furthermore, at prometaphase Ranjan et al. show just a single sister centromere pair, without showing the entire nucleus (or indeed a GSC marker) and it is not clear how they discriminate each sister centromere-pair. As we explain in our methods section, we used the combination of the marker H3T3P and the Z-stack to define each single pair (in which each centromere focus is very clear to distinguish given the high resolution of our images) and we present all

images captured in a very transparent manner. Given that the fluorescence of centromere foci can vary between each other, even within a single nucleus, this might have influenced their quantification. To overcome this, we measure the total amount of CID per nucleus or in case of pro-metaphase/metaphase, the total amount on each side (GSC-side vs CB-side), so that this variability between single foci would not have a big impact on our quantification and final conclusion.

(iv) Finally, we believe our ratio of 1.2 in favour of GSCs is accurate as we consistently measure this value at different cell cycle stage (prometaphase, metaphase, S phase), but also among fly lines with different genetic backgrounds (wild type Oregon R, nanos-Gal4, H2Av-RFP CID-GFP at anaphase-not shown in the text due to the low number of nuclei considered). Furthermore, the same difference was found using three different microscopy techniques: super-resolution, confocal and widefield (with applied deconvolution) microscopy.

To reflect any similarities and explain any differences, we have added the following to the 'Discussion' on page 14, point (b):
Interestingly, in Drosophila male GSCs centromeric CAL1 is reduced between G2 and pro-metaphase [7], further suggesting a role for additional regulators of CID assembly, such as CYCA/B or HASPIN, at this time.

And on page 14, point (c):
In Drosophila male GSCs, an asymmetric distribution of CID on sister chromatids higher than 1.4 fold was reported [7]. This higher value might reflect distinct systems in males and females, or the quantitation methods used. Importantly, CID asymmetry in males is established in G2/prophase, in line with the time-window we define for CID assembly.

2. Related to the point above, it is quite striking that CID or Cal1 overexpression and Haspin knockdown results in an increased number of GSCs. This could be interpreted as disrupting an asymmetry to chromosome segregation, with a subsequent effect on cell fate. However, given the nature of these perturbations, it is important to use caution in interpreting these behaviors. For example, it is possible that these changes affect chromosome structure at non-centromere loci, altering the expression of key gene. Alternately, these perturbations may alter nucleolar function. Indeed, recent work from the Yamashita lab has pointed to an important role for rDNA and the nucleoli in key stem cell behaviors and possibly chromosome distribution. It would be helpful if the authors comment highlight these different possibilities, but also use caution in some of their interpretations.

This is a good point. We have added this possibility to the 'Discussion' on page 15 (point 2):
However, we cannot rule out that the effects on cell fate observed with our functional analysis might reflect alternative CAL1 functions outside of the centromere, for example due to changes in chromosome structure or gene expression.

3. The effect of Haspin on CID deposition is interesting, and to me unexpected, although H3T3 has been implicated in aspects of asymmetric cell division behaviors. However, it is not clear to me what the basis for this is. For example, Haspin could act through H3T3 phosphorylation, or could directly target Cal1 or another factor. The authors even use H3T3P as a marker, but don't correlate this with CID desposition or centromere asymmetries. Is it possible to monitor the relative timing and co-localization of the H3T3 mark and asymmetry with that of CID using their existing data? I would also appreciate it if the authors could speculate about the potential inhibitory role of Haspin.

For improved clarity, we have added an additional panel to Figure S2(M-Q) showing the timing of the appearance and colocalisation of H3S10P and H3T3P marks in GSCs in mitosis.
We now speculate on the potential role of Haspin in the 'Discussion' on page 14, last paragraph:
Interestingly, the time course of H3T3P appearance during the GSC cell cycle closely follows the timing of CID incorporation, suggesting that the asymmetric deposition of CID might drive the differential phosphorylation of the histone H3 on sister chromatids.

4. There are some sections of the text that would be much more accessible if the authors

were to streamline these substantially. For example, the first section on the cell cycle distribution of centromeres provides a nice demonstration of their ability to follow these events, but does not provide extensive impactful information that is critical to the conclusions in the paper. There are also sections where it is important for the authors to test the effect in their system, but where the result is not particularly surprising, such as the role of Cal1 in depositing CID.
5. There are also some wording choices that seem a bit odd. The repeated use of "Following" to start a sentence seemed awkward to me, for example.
To address points 4 and 5, we have made significant efforts to improve flow of the manuscript and hope that this has improved its readability in general.

Response to Reviewer #2:

Major points:
1) As a major point, I don't find that there is strong data supporting that CID assembly already starts in G2. The figures and the text refer to G2/prophase, not distinguishing them. On page 6 the authors themselves state that centromere assembly likely initiates during G2. Where is the data supporting this statement? Without providing further support, this statement later turns into the conclusion: "Our results confirm that similar to GSCs also neural stem cells assemble centromeres between G2 and prophase." To me it is still possible that CID assembly starts in mitosis with prophase. The authors should be able to distinguish CID loading in G2 vs prophase by using chromosome condensation, nuclear envelope breakdown and H3S10p as markers. Also, there is a noticeable increase in loading of CID from prophase to metaphase. The authors ignore this because it is not significant. However, at face value it looks not much different then increase between S phase and G2/prophase. Increasing the small N over 12 or 18 might easily give this result significance and would then change the conclusion. In other words loading could actually be happening from prophase to metaphase.
We thank the reviewer for raising this important point. Our data supports CID loading in G2 phase, but it also shows that CID loading initiates after DNA replication and continues into G2 phase until prophase, and possibly up to metaphase of mitosis. We have changed the description of results throughout the manuscript to reflect this possibility, focusing instead on our novel finding that CID loading in GSCs occurs after DNA replication and before chromosome segregation. For examples, in the first paragraph of the 'Discussion' on page 13: *Our analysis reveals that GSCs initiate CID incorporation after replication and that its deposition continues until at least prophase (Fig. 7H).*

2) The statement: CYCB depleted germaria seem to have more cells compared to the control (Fig. 2M-N')" is quite vague. This should be quantified.
We have now added this quantitation to page 7: *Similar to what has been previously described [51], we observed that CYCB depleted germaria have more cells compared to the control, by counting the number of VASA positive cells from a similar number of z-stack projections (nanos-Gal4=34.8±2.3 cells, n=21 germaria; CYCB RNAi=50.6±2.3, n=23 germaria, not shown; Fig. 2M-N').*

3) The authors use the Bam-Gal4 driver to knock down CAL1 and CID at later stages of egg development. The don't observe any effect on cell division but they also state that CID levels are not particularly diminished compared to the control. Isn't the most straightforward interpretation of this, that the knockdown simply didn't work? Instead the authors continue to conclude: "These results indicate that CID and CENP-C are already assembled at centromeres at this stage and CAL1 function is dispensable at least for the cell division occurring after the 8-cell stage."
This is a bold statement, that is currently not backed up by the data. Without proper quantification of CID levels (microscopy or Westerns?), this and the subsequent conclusion in the discussion should be revised.
We now provide evidence that the depletion of an additional centromere protein CENP-C was effective in germaria using the same *bam-Gal4* driver (shown in Figure S5S). We have modified this description of the 'Results' on page 12 as follows: *To confirm our knock down approach using the*

bam-Gal4 driver in germaria, we tested the functionality of the driver on another centromere protein (CENP-C). Our results (Fig. S5S) confirm effective CENP-C knock down at this stage.

Minor points:

Abstract
The first phrase in the abstract make for difficult reading. Better: "Centromeres are epigenetically defined by CENP-A-containing chromatin and essential for genome integrity."
We have changed this to*: Centromeres are epigenetically defined by CENP-A-containing chromatin and are essential for cell division.*

I find this statement and the use of the word "while" strange : ."..down or CENP-A over-expression drives stem cell self-renewal, while the CENP-A assembly factor CAL1 is crucial for cell division".
First, the paper shows that CAL1 o/e together with CENP-A also drives stem cell-renewal, and second is this to say that CENP-A is not crucial for cell division?
We have changed this to*: Importantly, symmetric incorporation of CENP-A on sister chromatids via HASPIN knock down or over-expression of CENP-A either alone or together with its assembly factor CAL1 drives stem cell self-renewal.*

Introduction:
Reference 25 is cited as BioRxiv but has since been published in Developmental Cell as is also referenced in this paper as 64. This reference should be updated.
Done.

The authors often speak of centromere assembly, when they actually mean CID (or CENP-A) assembly. This is confusing, as the centromere encompasses more than just CENP-A. Only later in the paper, when the binding of CENP-C and CAL1 also has been assessed does it make sense to use this more general term.
We have clarified this throughout the text, only using the term 'centromere assembly' when referring to both CENP-A and CENP-C assembly.

Again, the use of the word "while" as a conjunction doesn't make sense here and is confusing, when comparing Drosophila and human: "In flies, CID (the homologue of CENP-A) deposition requires activation of the anaphase promoting complex/cyclosome (APC/C) and degradation of CYCA 21, while centromere assembly is antagonised by Cdk1 activity and promoted by the kinase Plk1 in humans 29-31." - It suggests a contrast, even though CDK activity (and Cyclin B) also plays a role in antagonising CENP-A assembly in flies (also shown in this paper).
On page 3, we have modified this to read: *In flies, CID (the homologue of CENP-A) deposition requires activation of the anaphase promoting complex/cyclosome (APC/C) and degradation of CYCLIN A (CYCA) [21,29]. In humans, centromere assembly is antagonised by Cdk1 activity, while the kinase Plk1 promotes assembly [30–32]. Additionally, the CYCLIN B (CYCB)/Cdk1 complex inhibits the binding of CENP-A to HJURP, preventing CENP-A loading at centromeres [33].*

Page 4: typo: In Drosophila, CID binds to CAL1 (fly functional homologue of HJURP - missing")"
Done.

The connection between Cyclin B and Haspin should be introduced a little bit more detail already in the introduction with appropriate references (here it would be better to cite the original papers rather than the review (ref 54). Strangely there is no mention of CDK1, although Cyclin B is surely not acting alone.
On page 3, we have added the following: *Additionally, the CYCLIN B (CYCB)/Cdk1 complex inhibits the binding of CENP-A to HJURP, preventing CENP-A loading at centromeres [33].*

Results:

In this study spectrosome shape and positon plays an important role in distinguishing GSCs and CBs. It would be helpful to shortly mention here how it is recognized (antibody 1B1) and expand here or later what position goes with what cell type (the shape is explained a little later in the paper).

On the last paragraph of page 4, we have edited as follows: *The niche comprises the terminal filament and the cap cells. A cytoplasmic roundish structure called the spectrosome connects 2-3 GSCs to the cap cells (Figure 1A). The spectrosome is present in both GSCs and CBs and its shape can be used to define the cell cycle stage [37,38]. Upon asymmetric division, the daughter cell closer to the niche retains the "stemness", while the other, the CB differentiates and is detached from the niche together with its spectrosome.*

Typo: Page 5, top: To achieve our aim...

Done.

Page 7, top: The authors state: "This is different from CYCB localization pattern..." - but fail to explain in what way it is different.

We have edited as follows on page 6: *This is different from the CYCB localisation pattern, as it shows both cytoplasmic and nuclear localisation but fails to localise at centromeres (Fig. 2E-H and inset H').*

Page 7, top: Again, I would recommend to exchange "centromere assembly" with "CID assembly" because this is what is assayed.

We have changed this to: *Previous work showed that CENP-A assembly into centromeric chromatin is tightly linked to key cell cycle regulators (Stankovic et al., 2017).*

Page 7, top: Replace "Following" with "Next,..." here and elsewhere in the paper.

We have fixed this throughout the manuscript.

Not clear, why the authors decided to examine endoreplication as the next thing. Please explain.

To clarify, we have modified this statement on page 7 as follows: *Given that CYCA knock down can induce endoreduplication [52], we performed EdU staining on control and CYCA RNAi germaria.*

Page 8, bottom: For better understanding Figure 3 it would be helpful to mention in the text the correlation with the spectrosome, position. If I'm not mistaken this would be:... Following (better: Next), we measured the total amount of CID present on one set of chromosomes (spectrosome proximal) versus the other (spectrosome distal).

We have modified on page 8 as follows*: Using the position and orientation of the spectrosome, which has a round shape during mitosis [38], we specifically identified centromeres that will be inherited by the GSCs (spectrosome proximal) and centromeres that will belong to the CBs (spectrosome distal, Fig. 3A-D', Fig. S3A-N').*

Page 9: The paper uses a paragraphs and Fig 4 to show that CAL1 is required for cell proliferation an dCID and CENP-C recruitment. Is that surprising? I would expect that any one of these centromere proteins are important for proliferation.

Given what is already known about CAL1 function, we agree with the reviewer that this result is not particularly surprising. However, we believe it was necessary to perform these experiments in our system to confirm such results for GSCs as, to our knowledge, CAL1 depletion has not yet been performed in a stem cell system. We have modified our conclusion in on page 9/10 as follows: *This analysis confirms that also in stem cells, CAL1 is crucial for cell division and therefore also for differentiation.*

Page 9, middle: What is "enucleated"? Page 9, middle: The authors state: "To confirm that the microtubules enucleated were captured by centromeres, we also performed a staining for both tubulin and CID (Fig. Fig. 3J-K') -but the result is not described. Is it confirmed?

We apologise for this error. This result is confirmed. We have modified on page 9 as follows: *We also confirmed that the microtubules nucleated from the centrosome were captured by centromeres by performing a co-staining for both tubulin and CID (Fig. Fig. 3J-K').*

Page 10, top: Introduce the meaning of "agametic"
To explain better, we have replaced the word 'agametic' with: *CID knock down resulted in empty ovaries with no VASA-positive cells, and therefore no germ cells (Fig. S4A).*

Page 11: "We first quantified the number of round spectrosomes (Fig. 5A-D and M)..." - And that is indicative of what?
We have replaced with: *We next quantified the number of round spectrosomes, using antibody staining against 1B1, indicative of GSC and CB cells (Fig. 5A-D and M).*

Page 12, middle:
I do not understand this statement: "In order to investigate whether asymmetric inheritance of CID between GSC and CB has a role in the stem cell niche disruption (Fig. 5)." - Isn't the asymmetric inheritance the normal situation? Why "disruption"?
We apologies for any confusion. We have replaced the word 'disruption' as follows on page 11: *To investigate whether the asymmetric inheritance of CID between GSC and CB has a role in regulating the stem cell asymmetric division (Fig. 5),…*

On page 12: With the given order of the paragraphs, I get the impression the story is told backwards. After increasing the CID levels (by overexpression alone or with CAL1, or Haspin knockdown), wouldn't it first make sense to analyse CID levels (symmetric vs. asymmetric) before looking at the effect on cell fate?
Our logic was to link the timing of CID loading (after between replication, but before chromosome segregation) to any asymmetric distribution of CID on sister chromatids. We have added an opening sentence to clarify this on page 8: *To explore whether the timing of CID assembly might be linked to an asymmetric distribution of CID on chromosomes, we investigated CID distribution on sister chromatids in GSCs prior to division.*

End of Results, just before Discussion: The last conclusion should be rephrased for better understanding (assuming the statement is correct, see Major comment 3).
Given our inclusion of additional RNAi data providing support for the efficient depletion of CENP-C at this stage, we have modified this sentence to read: *Taken together with our observation of no significant reduction in CID after CID or CAL1 RNAi at this stage (Fig. 6 and S5), these data suggest that CID is inherited from the GSCs with little new CID loading occurring in cysts.*

Discussion:
Page 15: I find the link to the Swartz et al. paper a little far-fetched. I don't see any particular logical connection between small differences in CENP-A levels determining cell fate, to small incremental loading of CENP-A which amounts to larger amounts of CENP-A over time.
We have modified this statement on page 14 (point 1c) to link a gradual CID loading to a particular cell type with a specialised function: *We observed that a 1.2-fold difference in CID and CENP-C levels between GSC- and CB-chromosomes can bias segregation. While this difference is small, it fits with the observation that small changes in CENP-A level (in the order of 2-10% per day) impacts on centromere functionality in the long run* [25].

In turn, I'm really surprised that the work of the Lampson lab is not cited in this paper, which provides support to the centromere drive hypothesis and similar to this study concludes that kinetochore strength has an impact on chromosome segregation and cell fate.
We thank the reviewer for pointing out this oversight. We now refer to this paper in the Discussion (point 1c): *Finally, our results are in line with findings that the long term retention of CENP-A in mouse oocytes has a role in establishing asymmetric centromere inheritance in meiosis* [69].

.

Methods:

EdU: It should give more information and say: Ovaries from young female flies were dissected...

Done.

Figure 1: The red writing in G and H is hard to read. Also, the white EdU and green CID are hard to distinguish.

In general, throughout the manuscript, we have coloured the spectrosome (1B1) in red and cell cycle markers such as EdU in white. For consistency, we would like to keep this colour scheme. We show the CID channel only in panels GV and GH of Figure 1 to allow visualisation of the CID signal more clearly.

January 9, 2020

RE: JCB Manuscript #201910084R

Dr. Elaine Dunleavy
National University of Ireland Galway
Biosciences
Dangan
Galway 0000
Ireland

Dear Dr. Dunleavy:

Thank you for submitting your revised manuscript entitled "Asymmetric assembly of centromeres epigenetically regulates stem cell fate". We have now assessed the revised paper and would be happy to publish it in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

In addition to the formatting and style-related changes indicated below, we also feel that you need to modify the text slightly to make the reasoning for knocking down CENP-C using the Bam-Gal4 driver somewhat clearer to the reader. For example, you may say something along the lines of: "Since CID levels were not decreased after expression of RNAi using the Bam-Gal4 driver, we sought to confirm this knockdown approach in germaria. Therefore, we tested the functionality of the driver on another centromere protein (CENP-C). Our results (Fig. S5S) confirm effective CENP-C knock down at this stage. In addition, since other drivers successfully knocked down CAL1 and CID, this observation supports the idea that at this stage CID and CENP-C are already assembled at centromeres and that CAL1 function is dispensable, at least for the cell division occurring after the 8-cell stage."
Of course, you may phrase this as you like so long as it makes the logic as clear as possible to the reader.

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

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Full guidelines are available on our Instructions for Authors page, http://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

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2) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel

electrophoresis.

3) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (both in the figure legend itself and in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, since you used parametric tests in your study (e.g. t-tests, ANOVA, etc.), you should have first determined whether the data was normally distributed before selecting that test. In the stats section of the methods, please indicate how you tested for normality. If you did not test for normality, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

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6) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. imaging medium
e. Fluorochromes
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g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

7) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

8) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles/Tools may have up to 5 supplemental figures. At the moment, you are below this limit but please bear it in mind when revising.
Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section.

9) eTOC summary: A ~40-50 word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person.

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

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Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

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

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