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Suv420 enrichment at the centromere limits Aurora B localization and function

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MS TITLE: H4K20 methylation modulates Aurora B localization at centromeres and regulates mitotic fidelity

AUTHORS: Conor P Herlihy, Nicole M Hermance, and Amity Manning
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

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

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

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

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

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to

all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

Many cancers display chromosome number instability, and understanding the sources and consequences of this instability is therefore important. Herlihy et al. investigate the effect of increasing the concentration of H3K9 and H4K20 methylases at centromeres on Aurora B localisation and chromosome segregation in cell lines. They find that increased H3K9me3 and H4K20me3 correlate with loss of Aurora B localization at inner centromeres and with error prone chromosome segregation. In addition, they show evidence that the probability of survival decreases when the expression of histone demethylase KDM4A is low, or the expression of Suv39h2 or Suv420h2 methyltransferases is high in individuals with certain cancers. They conclude that increased H3K9me3 and H4K20me3 are potential sources of chromosome instability in cancer and they suggest that this may be due to defects in cohesion and Aurora B localization.
Overall, the paper provides interesting evidence that increasing H3K9me3 and H4K20me3 at (ill-defined)
regions at centromeres can alter the recruitment of Aurora B. However, the mechanism of this effect is unclear, and it remains speculative whether cohesion changes or Aurora B delocalization underlie the observed defects in chromosome segregation. Finally, I was left unconvinced that previous studies really support a role for increased pericentromeric histone methylation in cancer, and the cancer and cell line database analyses provided here appear to lack rigor.

Comments for the author

Major points
1. Is there really prior support for the idea that increases in these histone marks are found in cancer? In the Introduction, it is stated that Taylor et al. (2018) find that “expression of Suv39 and Suv420 … correspond with levels of aneuploidy”. I may have missed it, but I could not find anything in this paper to support this idea. The study analyses mutations, not expression levels, and the inverse correlation of mutations in Suv39 and Suv420 genes with aneuploidy was not significant ($p = 1$, Figure S3). In addition, the review by Janssen et al. cited in the Results is overwhelming concerned with loss of heterochromatin marks in cancer, not gain. I struggled to find anything to support the idea that there are global (or pericentric) increases in these marks in cancer.

2. How were the cancers analysed in Fig 1 and S1 chosen? This is potentially a critical question. Are there other cancers where histone methyltransferase/demethylase expression and survival do not correlate in this way? If so, then whether the cancers to be analysed were selected randomly, and blindly, prior to the analysis becomes vital to know. As there are numerous cancers that could be examined in this way, there will be a significant false discovery rate. How was this taken in to account?

3. In many cases, control experiments are labelled “mock”. I cannot find a description of what this means but, for many experiments, a “mock” treatment in the usual sense may not be an adequate control. For example, it is conceivable that expressing CENP-B-GFP alone could alter centromere function, so experiments expressing CENP-B-Suv-GFP proteins should have CENP-B-GFP controls.

4. As is implied in the paper, altering KDM4A expression levels is likely to alter many aspects of cell biology beyond the function of pericentromeric heterochromatin, including the expression of multiple genes that may be influenced directly or indirectly by heterochromatic marks. Does CENP-B-based targeting of Suv39 or Suv420 also increase alignment errors in the Monastrol assay?

5. Ideally, the individual data points should be shown on graphs, not just the means and SD. It should be made clear if statistical tests used n = 3 biological replicates, or the n included multiple cells or kinetochores.

It would be easier if these n numbers were given in figure legends, not just in the methods.

6. The IF images in Figure 2 are too small to see where H3K9me3 and H4K20me3 are targeted. Can larger images or, better, chromosome spreads, be shown to make this clear? A reasonable expectation is that the new induced methylations will be found in patterns similar to that of CENP-B (in chromatin beneath kinetochores). However, this pattern would be rather at odds with the model shown in Figure 6, where increased H3K9me3 and H4K20me3 are shown encroaching from pericentromeric heterochromatin. Does this model really match the data?

7. It is surprising that previous studies targeting H3K9 methyltransferases to centromeres in HACs are not discussed here (eg Ohzeki et al. EMBO J. 2012;31:2391–2402; Martins et al MBoC 2016;27:177–196).

8. Ohzeki et al. 2012 argue that tethering Suv39h1 to centromeres prevents CENP-A assembly. Were CENP-A quantities on chromosomes assessed here? If not, can we be sure that using ACA (of which CENP-A is a major component) for normalisation is valid?

9. In Figure 4, staining for total CENP-A and total Hec1 is needed to know if phosphorylation of these components is specifically altered, particularly in light of point 8 above.

10. The results in Figure 5E and S3F are interesting, but I am interested to know more about the compounds selected for these graphs, and the statistical significance of these results in what is essentially a large multiple comparison analysis (because there are multiple inhibitors in this database). I could not find any information about the selectivity profile of Cpd-10. Does it prefer AurA or AurB? Does it hit kinases other than Aurora kinases? Without this information, the results are not very informative. ZM-447439, which is a selective AurB inhibitor (https://doi.org/10.3389/fonc.2015.00285) is present in the GDSC1 dataset, but not included here. Why is this? Also, Tozasertib inhibits both AurA and AurB, as well as other kinases, and causes AurB-inhibition-like phenotypes in cells, so it might be a complicated case. In contrast, more AurA-selective inhibitors like MLN-8237 that are present in the GDSC1 database are not shown. Why was the Mps1/TTK inhibitor Mps1-In1 selected for display, but not TTK-3146 (which I assume is aka AZ3146)?

Minor points

1. It is odd to superscript methylation states (eg me3), when the standard and widely used nomenclature system does not.
2. Care needs to be taken in the phrasing of introductory material. While kinetochores assemble on regions that are generally heterochromatic, the core centromere regions ("centrochomatin") may be less heterochromatic. Indeed, nucleosomes containing the CENP-A variant of H3 are unlikely to carry the classic pericentromeric heterochromatin mark H3K9me3.
3. In the Introduction, it is stated that loss of Aurora B from centromeres stabilizes microtubule attachments, but this is actually rather controversial, with some researchers arguing that kinetochore- or microtubule-bound Aurora B is more important.
4. The original reference for Kmplot.com should be provided.
5. It is stated that "all" chromosome attachments are syntelic upon monastrol treatment. Is this really true?
6. In the Discussion, it is suggested that HP1 may act by "promoting … Aurora B localization (through recruitment of Suv420 and increased H4K20me3)". I found this confusing, because the model is apparently that H4K20me3 generates a chromatin state to which Aurora B cannot bind.
7. The KDM4A RNAi reagents used are imprecisely described. What were the RNA regions targeted in which experiments? Was the control really a "scrambled" version of the same siRNA (ie the same nucleotides but their order rearranged")?

Reviewer 2

Advance summary and potential significance to field

In this manuscript the authors describe a potential role for H3K20 methylation in the regulation of centromeric localisation of the mitotic kinase Aurora B

(ABK) and aim to correlate this to survival probability of cancer patients with high levels of the methyltransferases that indirectly (Suv39h2) or directly (Suv420h2) regulate H4K20 methylation, and with patients expressing low levels of the demethylase KDM4A.
Whilst the observation that Aurora B becomes displaced from centromeres when Suv420h2 is forced at centromeres is interesting, the lack of any mechanistic insight as to why increased methylation of H4K20 would displace ABK and to what extend endogenous Suv420h2 and H4K20Me3 are involved in ABK regulation, makes it difficult to assess if this observation is of true physiological relevance. Moreover, contradicting literature is often not acknowledged or discussed, and the link to cancer patient survival appears far-fetched.

Comments for the author

Major points:
1) In Figure 1A-C and S1A low expression of KDM4A, and high expression of Suv39h2 or Suv420h2 correlates with reduced (lung, breast, renal) cancer free survival. The authors subsequently refer to Taylor et al. Cancer Cell (2018), to claim that increased Suv39 or Suv420 expression corresponds to the degree of aneuploidy in cancer samples. The latter is a bit of an overstatement. Based on Table S5 of Taylor et al (2018), there appears to be a weak correlation between aneuploidy and Suv39 and Suv420 expression. Furthermore, forcing high amounts of Suv39 or Suv420 near centromeres does most likely not reflect what happens in patient cells expressing high levels of these enzymes, nor prove that these enzymes have to be at centromeres to exert their function (as suggested in the text). Does mere overexpression of Suv39 and Suv420 affect the centromere levels of H3K9Me2/3, H4K20Me3, and Aurora B? And does it affect chromosome segregation fidelity? If mere overexpression affects both, can the segregation fidelity be rescued by re-localizing ABK back on the centromere through expression of CenpB-INCENP?

2) The authors claim that the (very mild) segregation defects caused after knockdown of KDM4A and by cen-Suv39 overexpression are due to enhanced (HP1-dependent) Suv420 localization and increased H4K20Me3 at centromeres. However there is no evidence for this. In fact, cen-Surv39 expression appears to increase H3K9me2 and H4K20me3 all over the chromosomal arms and not specifically at centromeres (fig 2A). Moreover, according to their model, knock-down of KDM4A should enhance centromeric H4K20Me3. The authors should substantiate this claim by measuring the levels of H4K20Me3 after KDM4A knockdown and testing if partial knockdown of Suv420 in cen-Suv39 expressing cells rescues alignment defects.
Also, there is quite some evidence that centromeric levels of HP1 in mitosis are not dependent on H3K9Me2/3 but are recruited by INCENP (Hirota et al 2005 Fischle et al. 2005, Kang et al MBoC 2011, Abe et al. Dev Cell 2016). It is unclear why these data are not discussed as they have implications for the proposed models.

3) The effect of cen-Suv420 expression on levels of centromeric levels of H4K20me3 and Aurora B seems very clear. However, the authors chose to analyse metaphase cells, when ABK centromere levels (and pHec1 levels) are already significantly lower (especially in RPE1 cells, see Salimian et al, Curr Biol 2011, DeLuca et al. JCS 2011). Are centromere levels of ABK in prometaphase (or for instance after Eg5 inhibition or nocodazole treatment) also affected by cen-Suv39 or cen-suv420 overexpression, or is the effect only seen during metaphase?

4) Cen-Suv420 expression causes alignment and segregation defects described for ABK inhibition. Preferentially (but maybe too much to ask) it should be shown that these alignment/error correction defects can be rescued by restoring centromere localization of ABK through low level expression of CenpB-INCENP.

5) Lack of any mechanistic insight as to why high levels of H4K20Me3 would displace ABK from centromeres is the main concern. Some hypotheses are proposed in the discussion (page 11), which can be easily tested. For instance, the authors hypothesise that extra H4K20Me3 increases cohesion and persistent centromeric transcription. Inter-kinetochore distances can be measured in the cen-Suv420 expressing cells to figure out if cohesion is indeed increased.
Moreover, they could test if inhibition of PolII using triptolide can rescue the alignment/segregation defects induced by cen-Suv420 expression.

6) Based on RNAseq expression data of the cancer cell line encyclopedia and drug sensitivity data by Welcome Sanger Institute the authors predict that cell lines with high expression of Suv39 and Suv420 exhibit increased sensitivity to drugs that target ABK but not to drugs that target Mps1 or AAK. Importantly there appears to be a trend in sensitivity to AAK inhibition (Figure S3F Tozasertib). This most likely does not reach statistical significance because the number of data points is way less than in the other figures. Furthermore these cancer cell lines with high expression levels of Suv39 and Suv420 seem excellent tools to substantiate their conclusions. Centromere levels of H3K9Me2/3, H4K20Me3 and Aurora B should preferentially be quantified in these cell lines and compared to cell lines with lower expression levels.
Additionally, sensitivity for the different ABK, AAK and Mps1 inhibitors should have been tested in the RPE1 cell lines expressing cen-Suv39 and cen-Suv420

Other points:
In general referencing is a bit careless
- Page 5: Fusion of the DNA binding domain to a protein of interest to mediate centromere targeting of that protein, was first described by Pluta et al, JCB 1992. This reference should be included.
- Page 6: Consistent with published work showing that H3K9 methylation helps to promote H4K20 methylation …. Please include references of this published work.
- Page 7: Description of the monastrol wash-out assay to asses error correction efficiency…. refer to Lampson and Kapoor. Nat Cell Biol. 2004.
- Page 7/9: functional inactivation of ABK kinase activity ….. Please refer to the original data papers (Kallio et al Curr Biol 2002; Hauf et al. JCB 2004, Ditchfield et al JCB 2003) and not to reviews.
- Page 8: Each of these histone phosphorylation events is independently sufficient to recruit Aurora B to centromeres… Include: Hadders et al JCB 2020.
- Page 11: …as well as positive regulation by phosphorylation of H3T3 and H2AT120.. Please refer here to the original papers that demonstrated this (Kelly et al, Science 2010, Wang et al, Science 2010; Yamagishi et al Science 2010 Tsukahara et al., Nature 2010).
- Page 11: ….demonstrated that enrichment of centromere and pericentromere cohesion is sensitive to Suv420 increases in H4K20Me3 levels. Manning 2010 does not demonstrate this, only Manning 2014 and Hahn et al 2013 do.
- Page 11: Other studies show that increased cohesion allows for persistent transcription of mitotic centromeres and disruption of centromere localization of ABK.. Kleyman et al, 2014 does not show any of this and can be omitted. Also, I think the Perea-Resa paper is misinterpreted. It shows that increased arm cohesin after Wapl depletion causes ectoptic mitotic transcription most likely causing the redistribution of ABK from centromeres to arms.
Page 10: Chromosoome Passanger Complex, is misspelled. Should be: Chromosomal Passenger Complex.
Page 8: Changes in centromere methylation correspond with sensitivity to Aurora B inhibition in cancer cells….
The title of this paragraph does not reflect the content. There is no evidence of changes in centromere methylation based on the mRNAseq data.
Please indicate which phosphorylated residue is recognized by the pHec1 Ab. If it is S55, bear in mind that this site is not solely phosphorylated by Aurora B but also by Aurora A. (DeLuca et al. 2017).
There is no need to normalize the quantitative IF data (Fig 2 B-E; 4E-G, 5B-D S2D, S3B, C, E). Showing all individual data points (also for the controls) is much more informative, since it will show the number of measurements per conditions as well as the spread in the control group. It could explain why the increase in H3T3ph and H2AT120ph after knockdown of KDM4A or expression of cen-
Suv420 is quite large but not statistically significant (Fig. S3).
Please indicate the statistical test used in figure legends.
It would be very helpful to number the pages and figures.

Reviewer 3

Advance summary and potential significance to field

It is known that histone methylation is commonly dysregulated in cancers. The manuscript by Herlihy et al describes that over-enrichment of histone H3K9me2/3 and/or H4K20me3 at the centromere inhibits Aurora B localization at the centromere and suppresses Aurora B dependent phosphorylation at the kinetochore, leading to a failure in error correction of kinetochore attachment and to chromosome missegregation. The authors manipulated these histone methylation marks by two methods; siRNA-mediated depletion of H3K9 demethylase KDM4A, and artificial targeting of H3K9 methyltransferase Suv39 or H4K20 methyltransferase Suv420.
They demonstrate that these treatments increase levels of H3K9me2 and H4K20me3 while decrease Aurora B level at centromeres and phosphorylation of known Aurora B targets, CENP-A and Hec1. These treatments also increased the mitotic chromosome defects such as lagging chromosomes, while depletion of KDM4A, demethylase inhibited mitotic error correction, the processes which are dependent on Aurora B.
Since Suv420 is known to be recruited to H3K9me3 marks via HP1, and the effect of cen-Suv420-GFP on the Aurora B localization defect is much severer than cen-Suv39 the authors suggest that Suv420 acts downstream of Suv39 to inhibit Aurora B.

The impact of cen-Suv420-GFP expression on Aurora B localization looks interesting and surprising. Since this treatment did not affect known mechanism of Aurora B localization at the centromeres, such as H3T3ph and H2A T120ph, the study hints existence of a novel mechanism. While the results raise more questions than clarify existing questions, I find that these data have interesting implications in the future study. However, there are several concerns that must be clarified before publication.

Comments for the author

Major points.
1. Page 4. “Global increases in H3K9me3 and H4K20me3 repressive heterochromatin marks have been described in several cancer contexts (Janssen et al., 2018, Black et al., 2012)”
These two cited review papers report several cases where these methylation processes are suppressed in cancers, but I could not find any specific description that these marks are globally increased in several cancers, as the authors imply.
It is commonly known that transposons are activated in cancer cells, likely reflecting the decrease of these heterochromatic histone marks. Please cite original research papers that report the global increase in these heterochromatin marks in cancers.

2. From method description, expression of cen-Suv39-GFP and cen-Suv420-GFP was done by transient transfection for some experiments and also by dox-induced expression of stably integrated gene in other experiments, but it is not clear which method was used for each specific experiment. This must be explicitly stated.
Please specify which Suv39 and Suv420 isoforms (h1 or h2?) were used for centromere targeting.
Since Suv39 and Suv420 accumulate at the inner centromeres and pericentromeres, the negative control of Suv39-GFP and Suv420-GFP must be done to show that the localization of cen-Suv39-GFP and cen-Suv420-GFP is dependent on CENP-B. Also, in Figure S2E, compare the expression level of the fusion proteins with endogenous proteins. If the expression levels of the fusion proteins are massively higher than endogenous levels, it would be difficult to conclude that the observed phenotypes are caused by centromere targeting.

3. Figure 2A. The data show that the increase of H4K20me3 is induced by targeting Suv39 (by cen-Suv39-GFP) or Suv420 (by cen-Suv420-GFP) to centromeres.
However, in the case of cen-Suv39-GFP, there is a massive increase of H3K9me2 not only on centromeres but also on chromosome arms. The similar increase of H3K9me2 on chromosome arms can be seen with cen-Suv420-GFP. One of the common issues of IF staining with H3K9me2/3 staining is that the antibodies against these methyl marks do not react when the adjacent H3S10 is phosphorylated. If cen-Suv420-GFP indeed decreases Aurora B activity, this may increase H3K9me2 reactivity by decreasing H3S10ph levels. To resolve these confusions, it would be important to test if H3K9me2 antibody can be affected by H3S10ph, and also if cen-Suv420-GFP decreases H3S10ph levels. Authors should explain why this increase of methylation level at chromosome arms is induced.
In addition, chromosome architectures of some representative images look abnormal

(cen-Suv39 with H3K20me3 staining, cen-Suv420 with H3K9me2 staining). The method implies that the authors did not fix the samples at all for this analysis. The authors should provide rationale behind this procedure, and discuss whether the change in apparent chromosome morphology between samples could affect IF staining patterns.
Statistical analyses do not appear to match with representative images.

4. Page 6, “Enhanced H4K20me3 compromises mitotic error correction mechanisms” and Figure 3, “Mitotic error correction is compromised when H3K9 methylation levels are increased”. The experiments shown in Fig. 3 are solely based on KDM4A depletion by siRNA.
Figure S2C showed that this treatment mildly increased H3K9me2 levels at centromeres, but never shown the effect on H4K20me3. The authors should monitored H3K9me2, H3K9me3, and H4K20me3 levels upon siKDM4A treatment by both IF and western. The authors should also change the titles for this section and the figure to better reflect the actual experiments.
It is not clear if these experiments were performed with a pool of siRNAs or just one siRNA. It is a standard in the cell biology field that siRNA specificity is confirmed by expression of siRNA-resistant mutant. Reliance on the phenotypic analysis by a pooled siRNA is not acceptable.

5. Figure 3C. Standard information behind statistics is missing. Chromosome misalignment can be caused by unstable kinetochore attachment or hyper-stable attachment. This can be assessed by cold resistance of microtubules and/or Mad2 staining.

6. Effect of cen-Suv420-GFP on Aurora B localization is striking. Since CPC formation is critical for Aurora B localization and activation, it would be important to check total expression levels and localization of the CPC subunits.
It is also standard to examine if the CPC formation is affected by cen-Suv420-GFP using immunoprecipitation.

7. The title “H4K20 methylation modulates Aurora B localization at centromeres and regulates mitotic fidelity” is not accurate. The authors show that Suv420 enrichment at the centromere interferes with Aurora B localization, but it remains to be tested if H4K20 methylation is important. It is possible that the critical target of Suv420 is not H4, and such a possibility should be discussed.

Minor comments
1. Methods for fluorescent signal normalization are missing.
2. ‘Anaphase defects’ are not well defined. An example shown in Figure1H do not show the typical lagging chromosome phenotype. As an alternative measure for chromosome segregation defect, frequency of micronuclei would be informative.
3. In this paper, all statistical analyses are done by Student’s two tailed t-test. Students’ t-test has to be applied only to the ‘normally distributed’ data sets. It would be important to show data distribution, and justify the usage of this statistical analysis. Also, describe the number of analyzed samples/experiments for each panel.
4. Page 14. H3K9me3 must read H3K9me2.
5. Hec1 has multiple phosphorylation sites. Please specify which phosphorylation site was monitored. The pHec1 antibody apparently stains at the regions devoid of chromosomes, and not specifically at the kinetochores in Mock sample. How do you know the specificity of the antibody? In Figure 5C, pHec1 signals are massively accumulated at the centrosome when they expressed cen-Suv420-GFP compare to Mock. Is this reproducible?
6. Figure 2A. Mock treatment images on upper right panel look fuzzier than other images. Please confirm that those images were correctly deconvoluted.
7. Figure 4B-D. Define AFUs, and what does the value 1.0 means. The AFU values in B-D do not match to AUC AFUs shown in E-G. What does AUC AFU mean here, and how were the values calculated? Also, define it in Figure 2B-E.
8. Figure 5E. Please define IC50 AUC.
9. Please specify the version of R programming environment and plugin for statistical analysis used in patient survival data analysis.
10. Add an abbreviation for HR in Figure 1A-C and S1as hazard ratio.

First revision

Author response to reviewers' comments

Response to reviewer comments

Reviewer 1
Advance Summary and Potential Significance to Field:
Many cancers display chromosome number instability, and understanding the sources and consequences of this instability is therefore important. Herlihy et al. investigate the effect of increasing the concentration of H3K9 and H4K20 methylases at centromeres on Aurora B localisation and chromosome segregation in cell lines. They find that increased H3K9me3 and H4K20me3 correlate with loss of Aurora B localization at inner centromeres and with error prone chromosome segregation. In addition, they show evidence that the probability of survival decreases when the expression of histone demethylase KDM4A is low, or the expression of Suv39h2 or Suv420h2 methyltransferases is high in individuals with certain cancers. They conclude that increased H3K9me3 and H4K20me3 are potential sources of chromosome instability in cancer, and they suggest that this may be due to defects in cohesion and Aurora B localization. Overall, the paper provides interesting evidence that increasing H3K9me3 and H4K20me3 at (ill-defined) regions at centromeres can alter the recruitment of Aurora B. However, the mechanism of this effect is unclear, and it remains speculative whether cohesion changes or Aurora B delocalization underlie the observed defects in chromosome segregation. Finally, I was left unconvinced that previous studies really support a role for increased pericentromeric histone methylation in cancer, and the cancer and cell line database analyses provided here appear to lack rigor.

Response: We thank reviewer one for their thoughtful comments
Reviewer 1 Comments for the Author:

Major Points

1. Is there really prior support for the idea that increases in these histone marks are found in cancer? In the Introduction, it is stated that Taylor et al. (2018) find that “expression of Suv39 and Suv420 … correspond with levels of aneuploidy”. I may have missed it, but I could not find anything in this paper to support this idea. The study analyses mutations, not expression levels, and the inverse correlation of mutations in Suv39 and Suv420 genes with aneuploidy was not significant (p = 1, Figure S3). In addition, the review by Janssen et al. cited in the Results is overwhelming concerned with loss of heterochromatin marks in cancer, not gain. I struggled to find anything to support the idea that there are global (or pericentric) increases in these marks in cancer.

We thank the reviewer for raising this important point and agree that there is little in the literature that looks specifically at epigenetic marks at centromeres/pericentromeres. The repetitive nature of these genomic regions have precluded sequencing analyses used to study epigenetic mark distribution at other regions of the genome. Nevertheless, global changes in heterochromatin marks (or the enzymes responsible for their regulation) have been described in numerous cancer contexts. While many studies describe a relationship between a *decrease* in heterochromatin marks and tumorigenesis, other studies describe *increased* H3K9 and H4K20 methylation as promoting tumorigenicity. We have revised the introduction to more clearly describe this.

Lines 55-64: “While many studies have implicated changes in heterochromatin in the regulation of genome stability and cancer progression, most focus on the consequences of decreased H3K9me3 and H4K20me3 heterochromatin marks (reviewed in Janssen et al., 2018). Nevertheless, both increased expression of the methyltransferases responsible for placing these repressive marks, and decreased expression of demethylases that remove these marks, resulting in increased levels of H3K9me3 and H4K20me3, have been described in various cancer contexts (Janssen et al., 2018, Black et al., 2012, Zhou et al., 2019, Yokoyama et al., 2013). Functional studies in cancer cells indicate that these changes contribute to increased motility and metastatic potential (Zhou et al., 2019) (Yokoyama et al., 2013), and can limit therapeutic response (Cuellar et al., 2017, Guler et al., 2017).

We had previously cited a review by Janssen et al 2018 to support the idea that misregulation of epigenetic modifiers of heterochromatin marks may compromise genome integrity, and my own prior work (Black, Manning et al. 2012) showing KDM4A loss/decreased expression in cancer (22.1% of tumors) is similarly prevent to KDM4A gain/increased expression (18.9% of tumors). To better highlight prior work demonstrating a role specifically for increased H3K9me3 and/or H4K20me3 in tumorigenesis, we now also cite work by Zhou et al., 2019 describing increased H3K9me3 and H4K20me3 in esophageal carcinoma, and work by Yokoyama et al, 2013 describing increased H3K9me3 in breast and colorectal cancer, and work by Ohzeki et al., 2012 and Martins et al., 2016 describing the impact of tethered Suv39 to the centromere of a HAC on its mitotic segregation.

To complement these prior studies we also now include data from the TCGA showing both that the relative expression of Suv420 and Suv39 isotypes in various cancer subtypes is high, and that the expression of Suv39h1 and Suv420h2 is increased in nearly all cancer subtypes, compared to normal tissue. This new data is represented in Figure 1A.

Lines 104-107: “Expression data from the TCGA indicate that Suv39 and Suv420, the enzymes responsible for depositing methyl marks on H3K9 and H4K20, respectively, are highly expressed in cancer contexts, with Suv39h1 and Suv420h2 exhibiting increased average expression compared to corresponding normal tissue in eleven of thirteen cancer subtypes for which this paired tumor: normal data is available. (Figure 1A).”

Although not the main focus of the manuscript, in Taylor et al., 2018: “Genomic and Functional Approaches to Understanding Cancer Aneuploidy”, the authors do correlate expression data with degree of aneuploidy (presented in their Supplemental Table 5). These data show that Suv39h1, Suv39h2, and Suv420h2 expression each have highly significant positive correlations with degree of aneuploidy across all cancer types analyzed (aneuploidy score coefficient, and Bonferroni corrected p value: 0.241840095, p= 9.76E-175; 0.215680085, p=1.34E-122, and 0.18686464; 1.63E-83, respectively). We have confirmed with the primary author of this study that we have correctly interpreted this data.

Lines 69-72: “…recent work has revealed that expression of Suv39 and Suv420, the methyltransferases responsible for H3K9me2/3 and H4K20me2/3 respectively, correspond with levels of aneuploidy across 10,000+ cancer samples from the Cancer Genome Atlas Project (Taylor et al., 2018).”

We have now extended this observation by looking at the correlation between degree of aneuploidy and Suv39 or Suv420 expression in individual cancer subtypes. We find that this positive correlation is apparent and highly significant in 12/20 cancer subtypes represented in the TCGA. This data is presented in Figure 1B and Supplemental Table 1. CIN is a complex phenotype that is likely to be influenced by numerous factors. Therefore, we feel that though the correlation with aneuploidy is moderate, given the high significance, these correlations are consistent with our model whereby high Suv39 and Suv420 levels may be a contributing factor to mitotic defects and the generation of aneuploidy in cancer.

Lines 110-117:“Indeed, recent work describes that high expression of Suv39 or Suv420 positively correlates with pan cancer analyses of increased aneuploidy (Taylor et al., 2018). This relationship is not an artifact of high Suv39 or Suv420 expression in a single cancer type that happens to also be highly aneuploid but instead persists when samples are sorted by cancer subtype. We find that high expression of at least one Suv39 or Suv420 isoform demonstrate a significant positive correlation with aneuploidy in twelve out of twenty cancer contexts represented in the TCGA database (Figure 1B, Supplemental Table 1).”

2. How were the cancers analysed in Fig 1 and S1 chosen? This is potentially a critical question. Are there other cancers where histone methyltransferase/demethylase expression and survival do not correlate in this way? If so, then whether the cancers to be analysed were selected randomly, and blindly, prior to the analysis becomes vital to know. As there are numerous cancers that could be examined in this way, there will be a significant false discovery rate. How was this taken in to account?

We thank the review for pointing out this limitation to our initial analyses. Our original figures reflected cancer contexts where high H3K9me3 and/or H4K20me3 had been previously described, but as this reviewer points out, on its own these analyses provide minimal confidence in the relationship between expression and disease free survival, as we had not described this relationship more broadly. To address this concern, we now include a table (Supplemental Table 2) showing the Bonferonni corrected significance for the correlation between disease free survival and high expression of Suv39h1, Suv39h2, Suv420h1, and Suv420h2, across all cancer subtypes represented in the TCGA. These data indicate a negative correlation between disease free survival and high expression of Suv39h1/h2 or Suv420h1/h2 for four out of twenty cancer subtypes.

Lines 117-123: "Consistent with a correlation with aneuploidy, itself known to correspond with tumor aggressiveness and poor patient outcome (Pfau and Amon, 2012, Weaver et al., 2007, Davoli et al., 2013, Liu et al., 2016), high expression of a Suv39h1/h2 or Suv420h1/h2 isoform corresponds with a significant decrease in disease free survival in four out of twenty cancer subtypes represented in the GEO, EGA and TCGA databases (Figure 1C) (Nagy et al., 2018, Győrffy et al., 2010)."

We believe that, together, these new data provide additional evidence in support of a role for Suv39/Suv420 overexpression in the generation of mitotic errors that lead to aneuploidy, and is consistent with data demonstrating that aneuploidy and/or CIN correspond with tumor progression, drug resistance, and poor outcome.

3. In many cases, control experiments are labeled "mock". I cannot find a description of what this means but, for many experiments, a "mock" treatment in the usual sense may not be an adequate control. For example, it is conceivable that expressing CENP-B-GFP alone could alter centromere function, so experiments expressing CENP-B-Suv-GFP proteins should have CENP-B-GFP controls.

We have amended the figures and methods to re-label conditions as 'mock' and 'induced' and indicate that this terminology means the condition lacks or contains doxycycline addition/induction of expression constructs.

Lines 499-505: "Inducible expression was achieved by cloning the GFP tagged constructs into plvx-Tre3G-IRES (631362, Clontech) at Not1 / NdeI restriction cut sites….. Inducible expression of transgenes in was achieved by the addition of 2 μg/mL Doxycycline for 16-24h. "Mock" controls reflect the individual cell lines in the absence of doxycycline-induction of expression."

To better control for non-specific impairment of centromere function by targeted protein localization we now also include data from a control cell line showing induced expression of centromere-targeted GFP alone (cen-GFP) does not alter H4K20me3, or Aurora B localization at centromeres. These data are now represented in Figure 3B, Figure 4B, Supplemental Figure 3A, and discussed in the text.

Lines: 247-252: "Reduction in centromere localization of CPC components is dependent on centromere-enrichment of Suv420 as neither expression of centromere targeted GFP alone (cen-GFP) nor non-targeted Suv420 (Suv420-GFP) in RPE-1 cells is sufficient to both alter centromere H4K20me3 and disrupt Aurora B localization (Figure 3B, Figure 4B, and Supplemental Figure 3A)."

Lines 372-377: "…expression of centromere-targeted GFP, even at levels higher than that of cen-Suv39-GFP or cen-Suv420-GFP, is insufficient to alter centromere H4K20me3 or Aurora B localization (Figures 3B & 4B). Together, these data suggest that Aurora B localization and mitotic fidelity are not generally perturbed by protein tethering to the centromere but are instead specifically sensitive to Suv420 and/or H4K20me3 levels."

4. As is implied in the paper, altering KDM4A expression levels is likely to alter many aspects of cell biology beyond the function of pericentromeric heterochromatin, including the expression of multiple genes that may be influenced directly or indirectly by heterochromatic marks. Does CENP-B-based targeting of Suv39 or Suv420 also increase alignment errors in the Monastrol assay?

The reviewer raises an important point that we now address with new data showing that, similar to KDM4A depletion, overexpression of centromere-targeted Suv39 or Suv420 increases chromosome alignment defects following a monastrol washout assay. This data is represented in Figure 4 and discussed in the text. Importantly, these new data agree with our findings that Aurora B localization at centromeres is reduced, and the frequency of anaphase defects increased by targeting either Suv39 or Suv420 to the centromere.

Lines 219-224: "… KDM4A-depleted cells, as well as cen-Suv39-GFP and cen-Suv420-GFP expressing cells, exhibit a delayed progression to metaphase, with fewer than half of all cells able to achieve metaphase alignment within the same time frame (Figure 4A). Together, these data indicate that the mitotic error correction machinery is compromised under conditions where H3K9me3 or H4K20me3 is increased."

5. Ideally, the individual data points should be shown on graphs, not just the means and SD. It should be made clear if statistical tests used n = 3 biological replicates, or the n included multiple cells or kinetochores. It would be easier if these n numbers were given in figure legends, not just in the methods.

We thank the reviewer for these helpful suggestion and regret that the robustness of our analyses had not been adequately reflected in the original draft. We have replaced all bar graphs that reflect single cell or single centromere data with 'superplots' that reflect each individual data point. Replicates are color coded to allow visualization of both inter and intra- replicate variation. All measurements were performed on a minimum of 30 cells per condition, per replicate, and statistical tests reflect significance between biological replicates (n= 3). For clarity this information, as well as the number of individual measurements made per replicate, are now reflected in the figure legends as well as the methods section.

6. The IF images in Figure 2 are too small to see where H3K9me3 and H4K20me3 are targeted. Can larger images or, better, chromosome spreads, be shown to make this clear? A reasonable expectation is that the new induced methylations will be found in patterns similar to that of CENP-B (in chromatin beneath kinetochores). However, this pattern would be rather at odds with the model shown in Figure 6, where increased H3K9me3 and H4K20me3 are shown encroaching from pericentromeric heterochromatin. Does this model really match the data?

We have replaced the panels in Figure 2 (now represented in Figure 3B and Supplemental Figure 1D & E) with images of single chromosomes from metaphase spreads. We now include enlargements to better visualize the induced methylation changes. As the reviewer correctly suggests, in our experimental system where we target localization of the methyltransferase to the CENPB recognition sequence at centromeres we do observe enrichment of the corresponding methyl mark between ACA foci (at the centromere). The revised figure now better reflects this localization pattern.

The model in Figure 6 was meant to convey how we envision increased methylation in a cancer context (not via experimentally induced centromere localization of the methyltransferase *per se*) to impact Aurora B localization. We regret this confusion and have removed the graphic, focusing instead on the text to describe potential models. We now also highlight the similarities between our experimental tethering of methyltransferases to the centromere and how we predict overexpression of Suv420 in cancer may similarly impact Aurora B localization in the discussion.

7. It is surprising that previous studies targeting H3K9 methyltransferases to centromeres in HACs are not discussed here (eg Ohzeki et al. EMBO J. 2012;31:2391–2402; Martins et al MBoC 2016;27:177–196).

We thank the reviewer for alerting us to this unfortunate oversight- these citations have been added to our introduction and discussion where we now specifically discuss these supporting data.

8. Ohzeki et al. 2012 argue that tethering Suv39h1 to centromeres prevents CENP-A assembly. Were CENP-A quantities on chromosomes assessed here? If not, can we be sure that using ACA (of

which CENP-A is a major component) for normalisation is valid?

Contrary to Ohzeki et al., We do not observe changes in CENPA localization at centromeres. This data is now reflected in Supplemental Figure 5A, a distinction that is likely due to the short (24h) timeframe of our tethering experiments. We now discuss this possibility in the discussion.

Lines 341-357: "Work form other groups has described that a persistent increased in H3K9me3 limits CENPA deposition at the centromere on a human artificial chromosome (HAC), thus compromising centromere maintenance (Ohzeki et al., 2012, Martins et al., 2016, Martins et al., 2020). However, while induction of cen-Suv39-GFP of cen-Suv420-GFP for 24-28h (less than two cell cycles) is sufficient to cause dramatic changes in centromere levels of H3K9me3 and/or H4K20me3, in our system it appears insufficient to compromise centromere formation or maintenance as we detect no change in overall levels of CENPA at the centromere, or in the outer kinetochore protein Hec1 (Supplemental Figure 4, Figure 5). That the observed change following a short-term increase in Suv39 and/or Suv420 at centromeres is instead compromised CPC localization and reduced phosphorylation of Aurora B substrates suggest that spreading of the respective heterochromatin marks can impact centromere function in two distinct ways. First by subtly moderating regulation of microtubule attachments, and then more crudely by preventing centromere formation and maintenance. As methylation levels likely increase with duration of the experimental perturbation, whether these distinctions arise due to experimental differences in the total amount of H3K9/H4K20 methylation achieved at the centromere, or instead reflect a cumulative impact of persistent epigenetic deregulation over several cell cycles remains unclear."

Nevertheless, in our experiments ACA staining was not used for normalization and was instead used only to identify centromere/ kinetochore pairs for analysis. To account for technical variation in arbitrary fluorescence units between replicates, each measurement was normalized to the average fluorescence of the control condition for that replicate (ie mock induced, non- targeting siRNA, etc). Average values therefore reflect the relative change in fluorescence, not the absolute fluorescence intensity for each data point. We have changed the axes on graphs representing these data to "Relative Fluorescence" to better reflect that these values have not been normalized to a second fluorescent marker. Additional detail has also been added to the text and methods to clarify these points.

9. In Figure 4, staining for total CENP-A and total Hec1 is needed to know if phosphorylation of these components is specifically altered, particularly in light of point 8 above.

We now include data showing the total levels of CENPA and Hec1 remain unchanged following induced expression of centromere-targeted Suv39 or centromere-targeted Suv420. This new data is represented in Figure 5B and Supplemental Figure 4A.

10. The results in Figure 5E and S3F are interesting, but I am interested to know more about the compounds selected for these graphs, and the statistical significance of these results in what is essentially a large multiple comparison analysis (because there are multiple inhibitors in this database). I could not find any information about the selectivity profile of Cpd-10. Does it prefer AurA or AurB? Does it hit kinases other than Aurora kinases? Without this information, the results are not very informative. ZM-447439, which is a selective AurB inhibitor (https://doi.org/10.3389/fonc.2015.00285) is present in the GDSC1 dataset, but not included here. Why is this? Also, Tozasertib inhibits both AurA and AurB, as well as other kinases, and causes AurB-inhibition-like phenotypes in cells, so it might be a complicated case. In contrast, more AurA-selective inhibitors like MLN-8237 that are present in the GDSC1 database are not shown. Why was the Mps1/TTK inhibitor Mps1-In1 selected for display, but not TTK-3146 (which I assume is aka AZ3146)?

We thank the reviewer for this critique and for the prompt to further explore this data set. We have now assessed the drug sensitivity data for all 6 drugs in the GDSC that are characterized to inhibit Aurora kinase activity (A, B and/or C). In contrast to this reviewer's assertion that ZM-447439 is a specific inhibitor of Aurora B, this drug, along with Genentech CPD10 and GSK 1070916 are all described to inhibit Aurora A at a similar or lower concentration than Aurora B. Nevertheless, the sensitivity of cells with high Suv39 or Suv420 expression (top quartile) to these

drugs, as well as to Alisertib and CD532 (which preferentially target Aurora A) is increased compared to cells with lower Suv39 and Suv420 expression (bottom quartile). Sensitivity to a sixth Aurora inhibitor, Tozasertib, shows a similar trend but no significant correlation with Suv39 or Suv420 expression levels, likely due to the reduced number of cell lines for which both expression and drug sensitivity data were available. There were no drugs used in the GDSC that specifically target Aurora B kinase. We now also include analysis of the MPS1 inhibitor TTK-3146 (which is the same as AZ3146), together with MPS1IN1 and find sensitivity to these drugs do not correspond to Suv39 nor Suv420 expression. The statistical significance of these comparisons was determined using a students' two tailed t-test corrected for multiple comparisons using the Bonferroni correction method where $p < 0.00625$ is significant. These data suggest that cells overexpressing Suv39 and Suv420 are sensitive to inhibition of Aurora kinases, but can not implicate a specific sensitivity to Aurora B kinase inhibition.

To address the lack of Aurora B- specific inhibitors in the GDSC we also compared the impact of the Aurora B specific inhibitor Barasertib to that of Alisertib and MPS1IN1 in an assay to induce/monitor segregation errors. With this approach we see that, following induction of cen-Suv39-GFP or cen-Suv420-GFP expression, cells exhibit increased sensitivity (as indicated by an increase in anaphase lagging chromosomes) to both Aurora A and Aurora B inhibition. In contrast, cells with or without induced expression of Suv39 or Suv420 respond similarly to MPS1 inhibition. These new functional data (represented in Figure 6A and 6B) are consistent with results of our drug sensitivity correlation analysis described above and together support a model whereby Suv39/Suv420-dependent regulation of Aurora B kinase is functionally relevant to mitotic fidelity in cancer. These points are now reflected in the discussion.

Lines 467-475: "We find centromere tethering of either Suv39 or Suv420 is sufficient to compromise Aurora B kinase localization at centromeres and result in reduced phosphorylation of Aurora B substrates that are critical for proper chromosomes segregation. Hec1, a key Aurora B substrate that governs kinetochore microtubule stability and mitotic error correction, is also a substrate for the related Aurora A kinase (DeLuca, 2017). Consistent with this redundant regulation, our data indicate that when centromere methylation is experimentally enhanced, or in cancer contexts where Suv39 or Suv420 expression is high and H4K20me3 is therefore likely high, sensitivity to inhibition of both Aurora A and Aurora B kinase is increased (Figure 6)."

Minor points

1. It is odd to superscript methylation states (eg me3), when the standard and widely used nomenclature system does not.

We have updated the text and figures to remove superscripts to indicate methylation state.

2. Care needs to be taken in the phrasing of introductory material. While kinetochores assemble on regions that are generally heterochromatic, the core centromere regions ("centrochomatin") may be less heterochromatic. Indeed, nucleosomes containing the CENP-A variant of H3 are unlikely to carry the classic pericentromeric heterochromatin mark H3K9me3.

We have revised the introduction to more accurately reflect the distinct chromatin environments at the centromere and pericentromere.

3. In the Introduction, it is stated that loss of Aurora B from centromeres stabilizes microtubule attachments, but this is actually rather controversial, with some researchers arguing that kinetochore- or microtubule-bound Aurora B is more important.

We have revised the introduction to better reflect the localization and function of Aurora B both at centromeres and kinetochores.

4. The original reference for Kmplot.com should be provided.

We have now cited both Gyorffy et al., 2010 and Nagy et al., 2018.

5. It is stated that “all” chromosome attachments are syntelic upon monastrol treatment. Is this really true?

As the reviewer correctly points out, upon monastrol washout a large portion (but not all) kinetochore microtubule attachments are converted to merotelic attachments. We have revised the text to correct our phrasing.

Lines 209-212: “These spindles can not form amphitelic chromosome attachments and instead from syntellic or monotelic attachments. Upon washout of monastrol, a large portion of kinetochore attachments are converted to merotelic attachments (Kapoor et al., 2000, Lampson et al., 2004)”

6. In the Discussion, it is suggested that HP1 may act by “promoting … Aurora B localization (through recruitment of Suv420 and increased H4K20me3)”. I found this confusing, because the model is apparently that H4K20me3 generates a chromatin state to which Aurora B cannot bind.

We have revised these confusing statements to better describe HP1 as a binding partner that is shared by both Suv420 and Aurora B, and now discuss the possibility that competitive binding of Suv420 to HP1 may underlie the reduction in Aurora B centromere localization.

Lines 379-386: “HP1 directly binds the Aurora B-containing CPC through INCENP, and in doing so enhances the enzymatic activity of Aurora B (Abe et al., 2016, Kang et al., 2011). In turn, Aurora B-dependent phosphorylation of H3S10 limits HP1 association with H3K9 methylation (Fischle et al., 2005, Hirota et al., 2005), such that the interaction between HP1 and Aurora B both positively (through functional regulation) and negatively (through reduction of HP1 recruitment) regulates Aurora B activity at the centromere. Suv420 is similarly recruited to the pericentromere through interactions with HP1 and our data raise the possibility that CPC interaction with HP1 is limited when Suv420 is bound.”

7. The KDM4A RNAi reagents used are imprecisely described. What were the RNA regions targeted in which experiments? Was the control really a “scrambled” version of the same siRNA (ie the same nucleotides, but their order rearranged”)?

The control siRNA sequences used were horizon inspired cell solutions’ (formerly dharmacon) ON-TARGET plus non-targeting siRNAs. We have updated the methods, figures, and legends to better reflect this. Supplemental Table 3 includes all siRNA sequences used in this study.

Reviewer 2

Advance Summary and Potential Significance to Field:

In this manuscript the authors describe a potential role for H3K20 methylation in the regulation of centromeric localisation of the mitotic kinase Aurora B (ABK) and aim to correlate this to survival probability of cancer patients with high levels of the methyltransferases that indirectly (Suv39h2) or directly (Suv420h2) regulate H4K20 methylation, and with patients expressing low levels of the demethylase KDM4A. Whilst the observation that Aurora B becomes displaced from centromeres when Suv420h2 is forced at centromeres is interesting, the lack of any mechanistic insight as to why increased methylation of H4K20 would displace ABK and to what extend endogenous Suv420h2 and H4K20Me3 are involved in ABK regulation, makes it difficult to assess if this observation is of true physiological relevance. Moreover, contradicting literature is often not acknowledged or discussed, and the link to cancer patient survival appears far-fetched.

Response: We appreciate the reviewers’ candid critique and have addressed each individual concern as described below.

Reviewer 2 Comments for the Author:

1) In Figure 1A-C and S1A low expression of KDM4A, and high expression of Suv39h2 or Suv420h2 correlates with reduced (lung, breast, renal) cancer free survival. The authors subsequently refer to Taylor et al. Cancer Cell (2018), to claim that increased Suv39 or Suv420 expression corresponds to the degree of aneuploidy in cancer samples. The latter is a bit of an

overstatement. Based on Table S5 of Taylor et al (2018), there appears to be a weak correlation between aneuploidy and Suv39 and Suv420 expression.

We appreciate the reviewer's concern that the pan-cancer correlation between Suv39 and Suv420 with aneuploidy is 'weak'. However, consistent with previously reported relationships between these enzymes, our data indicate that Suv39 and Suv420 also share (at least partially) overlapping roles in the regulation of mitotic fidelity. As such, we expect that misexpression of any one of these enzymes (Suv39h1, Suv39h2, Suv420h1, or Suv420h2) could be sufficient to promote segregation errors and that the correlation of an individual enzyme with degree of aneuploidy would therefore be lower than if the function were served by a single enzyme.
Therefore, we feel that though the correlation is moderate, given the high significance, (aneuploidy score coefficient, and Bonferroni corrected p value: 0.241840095, p= 9.76E-175; 0.215680085, p=1.34E-122, and 0.18686464; 1.63E-83, for Suv39h1, Suv39h2, and Suv420h2, respectively), these correlations are consistent with our model whereby high Suv39 OR high Suv420 levels are sufficient to promote mitotic defects. This rationale is now discussed in depth in the discussion.

Lines 321-334: "Analyses presented here and previously reported by The Meyerson group (Taylor et al., 2018) indicate a moderate but highly significant correlation between degree of aneuploidy and the independent expression levels of Suv39h1, Suv39h2, Suv420h1, and Suv420h2. Consistent with previous reports, our experimental data indicate that these isoforms of Suv39 and Suv420 also share at least partially overlapping roles in heterochromatin regulation and mitotic fidelity (Tsang et al., 2010, O'Carroll et al., 2000). As such, we expect that misexpression of any one of these enzymes (Suv39h1, Suv39h2, Suv420h1, or Suv420h2) could be sufficient to promote segregation errors and that the correlation of an individual enzyme with degree of aneuploidy would therefore be lower than if the function were served by a single enzyme. Though the correlation with aneuploidy for each enzyme is moderate, given the high significance of this relationship, we propose these correlations are consistent with a model whereby high expression levels of a Suv39 or Suv420 isoform are sufficient to promote mitotic defects and contribute to the generation of aneuploidy."

Furthermore, forcing high amounts of Suv39 or Suv420 near centromeres does most likely not reflect what happens in patient cells expressing high levels of these enzymes, nor prove that these enzymes have to be at centromeres to exert their function (as suggested in the text).

We agree completely with the reviewer that our data do not demonstrate that Suv39 or Suv420 activity at non-centromere regions of the genome could not play a role in the correlation with aneuploidy and/or disease free survival. Instead, we propose our data suggest that enrichment of Suv39/Suv420 and their respective methylation marks at centromeres are *sufficient* to alter Aurora B localization and induce segregation errors. We have revised the results and discussion sections accordingly. To better reflect the diverse functions of Suv39 and Suv420 (and the implications of their misexpression) we have also added citations for several studies that implicate changes in these enzymes/their respective methyl marks, at non-centromere regions of the genome, in various aspects of tumorigenesis, including cell motility (Zhou et al., 2019, Yokoyama et al., 2013) and therapeutic response (Cuellar et al. 2017, Guler et al., 2017).

Lines 334-337: "While our data does not argue against the possibility that Suv39 or Suv420 function outside regulation of pericentromere heterochromatin may compromise genome stability in cancer, it does suggest that corruption of centromere regulation may be a contributing factor."

Does mere overexpression of Suv39 and Suv420 affect the centromere levels of H3K9Me2/3, H4K20Me3, and Aurora B? And does it affect chromosome segregation fidelity? If mere overexpression affects both, can the segregation fidelity be rescued by re-localizing ABK back on the centromere through expression of CenpB-INCENP?

We have previously shown that overexpression of Suv420 alone is not sufficient to significantly alter H4K20me3 at centromeric or non-centromeric regions (Manning et al 2014). Similarly, we now find that induced expression of (non-centromere targeted) Suv420 is not sufficient to alter

centromere methylation or Aurora B localization, suggesting that, in otherwise normal cells where Suv420 regulatory pathways remain functional, overexpression alone is insufficient to alter centromere/pericentromere regulation. Nevertheless, regulation of pericentromere/ centromere Suv420 localization may not be as tightly controlled in cancer contexts as we find a range of H4K20me3 levels at centromeres in a panel of breast cancer cell lines and, in some cases, an inverse relationship to centromere levels of Aurora B. These data are now represented in Figure 3, Supplemental Figure 3, Supplemental Figure 6, and described in the results and discussion sections.

Lines 247-252: "Reduction in centromere localization of CPC components is dependent on centromere-enrichment of Suv420 as neither expression of centromere targeted GFP alone (cen-GFP) nor non-targeted Suv420 (Suv420-GFP) in RPE-1 cells is sufficient to both alter centromere H4K20me3 and disrupt Aurora B localization (Figure 3B, Figure 4B, and Supplemental Figure 3A)."

2) The authors claim that the (very mild) segregation defects caused after knockdown of KDM4A and by cen-Suv39 overexpression are due to enhanced (HP1- dependent) Suv420 localization and increased H4K20Me3 at centromeres. However, there is no evidence for this. In fact, cen-Surv39 expression appears to increase H3K9me2 and H4K20me3 all over the chromosomal arms and not specifically at centromeres (fig 2A).

We thank the reviewer for this critique and the opportunity to clarify our experimental approach and interpretation. We had not meant to imply that the *only* change resulting from KDM4A depletion is an increase in Suv420 localization to centromeres. Indeed, as the reviewer points out, KDM4A depletion leads to an increase in H3K9me2/3 levels throughout the genome, *including* at the centromere. Indeed this was the major driving factor for our experimentation with centromere-tethered Suv39 and Suv420 to specifically increase methylation levels at the centromere.

Nevertheless, as the reviewer points out, we had regrettably neglected to quantify and report on the possible increase in H3K9 and H4K20 methylation along chromosome arms following KDM4A depletion, Suv39 expression, or Suv420 expression. We have now quantified total levels of chromatin-localized H3K9me3 (ie on arms + centromeres) and find that, as with our previous centromere-specific analysis, overall chromatin-localized H3K9me3 is increased following siKDM4A, cen-Suv39 or cen-Suv420 expression (Supplemental Figure 1E). However, a change in bulk H3K9me2 or H3K9me3 was not apparent by western blot analysis in mitotic (noc arrested) cen-Suv39 and cen-Suv420 expressing cells (Supplemental Figure 4), suggesting that expression of these centromere-targeted enzymes does not induce a widespread increase in methylation. Instead, these data are consistent with reviewer 3's suggestion that decreased Aurora B activity/decreased H3S10p 'unmask' the H3K9me2/3 epitopes making them more accessible to antibody staining.

Moreover, according to their model, knock-down of KDM4A should enhance centromeric H4K20Me3. The authors should substantiate this claim by measuring the levels of H4K20Me3 after KDM4A knockdown and testing if partial knockdown of Suv420 in cen-Suv39 expressing cells rescues alignment defects.

We have now measured H4K20me3 at centromeres of KDM4A-depleted cells (Figure 3B) and find that while we do see a consistent increase in H4K20 methylation across replicates, the increase at centromeres is not statistically different than that measured in control cells. These results are consistent with our other new data showing that overexpression of Suv420 that is not enriched at centromeres via a cen-fusion (Suv420-GFP) only induces a moderate increase in centromere levels of H4K20me3 (also Figure 3B). These data suggest that disruption of the centromere/pericentromere boundary may be key to the phenotypes we describe. We propose that increased expression of the relevant methyltransferases or decreased expression of the demethylase alone in 'normal' cells (like RPE-1 cells) is not sufficient to perturb centromere methylation levels and that additional regulatory steps, beyond misexpression of the relevant enzymes, must be altered to result in varied centromere H4K20me3 levels seen in cancer (Supplemental Figure 6).

Lines 360-366: “In otherwise normal cells, depletion of KDM4A, or overexpression of Suv420 alone causes only moderate changes in H4K20me3 at the centromere ((Manning et al., 2014), Figure 3B), suggesting that additional regulatory pathways may function to limit Suv420 enrichment or otherwise restrict H4K20me3 at centromeres. Nevertheless, this regulation of centromere/ pericentromere Suv420 localization may not be as tightly controlled in cancer contexts as we find a range of centromere H4K20me3 levels in a panel of breast cancer cell lines.”

Also, there is quite some evidence that centromeric levels of HP1 in mitosis are not dependent on H3K9Me2/3 but are recruited by INCENP (Hirota et al 2005, Fischle et al. 2005, Kang et al MBoC 2011, Abe et al. Dev Cell 2016). It is unclear why these data are not discussed as they have implications for the proposed models.

We agree that these studies have significant implications for our proposed model and thank the reviewer for pointing out our oversight in citing them. That HP1 directly binds the CPC through INCENP (Kang et al., 2011; Abe et al. 2016) and Aurora B-dependent phosphorylation of H3S10 ejects HP1 from its binding site on H3K9me3 (Fischle et al., 2005; Hirota et al., 2005) raises the possibility that increased Suv20, which similarly binds HP1 at the centromere, competes with the CPC for its interaction with HP1. We now include a more in-depth discussion of this intricate relationship between the CPC and HP1.

Lines 379-386: “HP1 directly binds the Aurora B-containing CPC through INCENP, and in doing so enhances the enzymatic activity of Aurora B (Abe et al., 2016, Kang et al., 2011). In turn, Aurora B-dependent phosphorylation of H3S10 limits HP1 association with H3K9 methylation (Fischle et al., 2005, Hirota et al., 2005), such that the interaction between HP1 and Aurora B both positively (through functional regulation) and negatively (through reduction of HP1 recruitment) regulates Aurora B activity at the centromere. Suv420 is similarly recruited to the pericentromere through interactions with HP1 and our data raise the possibility that CPC interaction with HP1 is limited when Suv420 is bound.”

3) The effect of cen-Suv420 expression on levels of centromeric levels of H4K20me3 and Aurora B seems very clear. However, the authors chose to analyse metaphase cells, when ABK centromere levels (and pHec1 levels) are already significantly lower (especially in RPE1 cells, see Salimian et al, Curr Biol 2011, DeLuca et al. JCS 2011). Are centromere levels of ABK in prometaphase (or for instance after Eg5 inhibition or nocodazole treatment) also affected by cen-Suv39 or cen-suv420 overexpression, or is the effect only seen during metaphase?

The reviewer makes an excellent point that phenotypes characterized during metaphase may be more pronounced in prometaphase cells, where control levels of Aurora B at centromeres is higher. To address this concern we have now complemented our original metaphase analysis with analysis of Aurora B levels at the centromeres of nocodazole-treated cells. We have completed these analysis in the presence and absence of induced cen-Suv39 or cen-Suv420 expression and find that while (as the reviewer points out) overall levels of Aurora B are higher, the same relative decrease in Aurora B localization at centromeres is apparent in cells with high Suv39 or high Suv420 at centromeres as we had reported in metaphase cells. These new data have replaced the original metaphase analysis in Figure 4B, and the metaphase analysis has now been moved to Supplemental Figure 3A.

4) Cen-Suv420 expression causes alignment and segregation defects described for ABK inhibition. Preferentially (but maybe too much to ask) it should be shown that these alignment/error correction defects can be rescued by restoring centromere localization of ABK through low level expression of CenpB-INCENP.

We agree with the reviewer that this is a very compelling question and we had hoped to address it by first inducing expression of a non-targeted Suv420 construct, followed by rescue with expression of a centromere-targeted INCENP fusion. However, overexpression of Suv420 alone, in the absence of centromere tethering, induces only a moderate increase in centromere methylation and does not perturb Aurora B localization (please also see response to point 1 above) precluding us from performing the suggested rescue experiment. However, to approach the question of whether decreased Aurora B localization is functionally relative in cells expressing

cen-Suv420 we instead explored the functional relationship between methylations changes and segregation errors by exploiting the deficiency we describe in Aurora B activity at the centromere. We now show that cells expressing the centromere-targeted Suv39 or Suv420 exhibit increased sensitivity to chemical inhibition of Aurora kinase activity, when compared to cells that do not express these constructs. This new data are now represented in Figure 6.

Lines 285-295: "Decreased CPC localization and increased rates of chromosome segregation errors may render cells exquisitely sensitive to further inhibition of Aurora kinases. To test this possibility, cells with and without induction of cen-Suv39-GFP or cen-Suv420-GFP expression were treated with inhibitors targeting Aurora B Kinase (Barasertib), the related Aurora A kinase (Alisertib), or the mitotic kinase MPS1 and monitored for mitotic progression. Consistent with reduced Aurora B localization and activity (Figures 4 & 5), while both control and cen-Suv39- GFP- or cen-Suv420-GFP expressing cells were similarly sensitive to MPS1 inhibition and high concentrations of Alisertib, we find that cells expressing cen-Suv39-GFP or cen-Suv420-GFP are susceptible to increased anaphase lagging chromosomes following short term treatment with low nanomolar concentrations of both Aurora kinase inhibitors, while control cells are not (Figure 6A & B)."

5) Lack of any mechanistic insight as to why high levels of H4K20Me3 would displace ABK from centromeres is the main concern. Some hypotheses are proposed in the discussion (page 11), which can be easily tested. For instance, the authors hypothesise that extra H4K20Me3 increases cohesion and persistent centromeric transcription. Inter-kinetochore distances can be measured in the cen-Suv420 expressing cells to figure out if cohesion is indeed increased. Moreover, they could test if inhibition of PolII using triptolide can rescue the alignment/segregation defects induced by cen-Suv420 expression.

To address this reviewer's critique, we have now analyzed centromere and pericentromere transcript levels in mitotic cells with and without induction of cen-Suv39 or cen-Suv420 and find that, consistent with spreading of heterochromatin, centromere tethering of either enzyme results in a dramatic decrease in alpha satellite transcripts. We present this new data in Supplemental Figure 6, and in the results and discussion connect this observation to previous reports that disruption of centromere transcription impairs Aurora B localization.

Lines 271-282: "Localization of the CPC is also sensitive to centromere transcription. Centromere transcripts have been shown to bind the CPC and regulate both CPC centromere localization and Aurora B activation (Jambhekar et al., 2014, Blower, 2016, Ideue et al., 2014). To test whether centromere transcription is altered by centromere tethering of Suv39-GFP or Suv420-GFP we measured levels of centromere transcripts in nocodazole-synchronized cells following 24 hours of cen-Suv39-GFP or cen-Suv420-GFP expression. While qPCR analysis shows expression of centromeric α satellite RNA is readily detected during mitosis, levels of each transcript were reduced by ~half in mitotic cells expressing cen-Suv39-GFP or cen-Suv420-GFP (Supplemental Figure 5C). These data suggest that suppression of transcription underlies defects in Aurora B localization when centromere levels of Suv39/H3K9me3 and/or Suv420/H4K20me3 are increased."

Lines 425-437: "This distribution of cohesin is required for transcription of mitotic centromeres such that disruption of either pericentromere cohesin enrichment, or transcription itself, impairs Aurora B localization (Jambhekar et al., 2014, Kleyman et al., 2014, Perea-Resa et al., 2020). Work from our group and others has demonstrated that Suv420-dependent H4K20me3 enhances pericentromeric cohesin (Hahn et al., 2013, Manning et al., 2014, Bernard et al., 2001). This relationship would predict that Suv420-dependent methylation would promote centromere transcription and Aurora B localization. However, we find that following Suv39/Suv420 tethering to centromeres, increased centromere H4K20me3 corresponds with reduced, not enhanced, transcription and Aurora B localization at centromeres. Together these data indicate that suppression of centromere transcription by spreading of H4K20me3 from the pericentromere into the centromere is sufficient, irrespective of cohesin enrichment, to disrupt CPC localization."

6) Based on RNAseq expression data of the cancer cell line encyclopedia and drug sensitivity data by Welcome Sanger Institute the authors predict that cell lines with high expression of Suv39

and Suv420 exhibit increased sensitivity to drugs that target ABK but not to drugs that target Mps1 or AAK. Importantly, there appears to be a trend in sensitivity to AAK inhibition (Figure S3F, Tozasertib). This most likely does not reach statistical significance because the number of data points is way less than in the other figures. Furthermore, these cancer cell lines with high expression levels of Suv39 and Suv420 seem excellent tools to substantiate their conclusions. Centromere levels of H3K9Me2/3, H4K20Me3 and Aurora B should preferentially be quantified in these cell lines and compared to cell lines with lower expression levels.

The reviewer makes an excellent point and we were eager to test our model in a panel of cancer cell lines. Though it proved challenging to locate and/or culture many of the cell lines represented in the drug sensitivity data set, we were able to obtain a small panel of five breast cancer cell lines with varying levels of centromere H4K20me3. We found that low H3K9me3 at centromeres did correlate with high Aurora B localization at centromeres and higher centromere H3K9me3 corresponded with lower centromere Aurora B localization. These data are represented in Supplemental Figure 6.

Additionally, sensitivity for the different ABK, AAK and Mps1 inhibitors should have been tested in the RPE1 cell lines expressing cen-Suv39 and cen-Suv420

Per the reviewer suggestion, we now show that cells expressing the centromere-targeted Suv39 or Suv420 exhibit increased sensitivity to chemical inhibition of Aurora kinase activity, when compared to cells that do not express these constructs. This new data are now represented in Figure 6 (see also Reviewer 2, comment 4 above).

Other points:

In general referencing is a bit careless
-Page 5: Fusion of the DNA binding domain to a protein of interest to mediate centromere targeting of that protein, was first described by Pluta et al, JCB 1992. This reference should be included.
-Page 6: Consistent with published work showing that H3K9 methylation helps to promote H4K20 methylation …. Please include references of this published work.
-Page 7: Description of the monastrol wash-out assay to asses error correction efficiency…. refer to Lampson and Kapoor. Nat Cell Biol. 2004.
-Page 7/9: functional inactivation of ABK kinase activity ….. Please refer to the original data papers (Kallio et al Curr Biol 2002; Hauf et al. JCB 2004, Ditchfield et al JCB 2003) and not to reviews.
-Page 8: Each of these histone phosphorylation events is independently sufficient to recruit Aurora B to centromeres… Include: Hadders et al JCB 2020.
-Page 11: …as well as positive regulation by phosphorylation of H3T3 and H2AT120.. Please refer here to the original papers that demonstrated this (Kelly et al, Science 2010, Wang et al, Science 2010; Yamagishi et al Science 2010,
Tsukahara et al., Nature 2010).
-Page 11: ….demonstrated that enrichment of centromere and pericentromere cohesion is sensitive to Suv420 increases in H4K20Me3 levels. Manning 2010 does not demonstrate this, only Manning 2014 and Hahn et al 2013 do.
-Page 10: Chromosoome Passanger Complex, is misspelled. Should be: Chromosomal Passenger Complex.

We have made each of the requested changes, including grammatical and spelling errors, replaced review articles with primary research articles and added additional references throughout.

-Page 11: Other studies show that increased cohesion allows for persistent transcription of mitotic centromeres and disruption of centromere localization of ABK.. Kleyman et al, 2014 does not show any of this and can be omitted. Also, I think the Perea-Resa paper is misinterpreted. It shows that increased arm cohesin after Wapl depletion causes ectoptic mitotic transcription most likely causing the redistribution of ABK from centromeres to arms.

We have the same interpretation of these studies and have revised the text to better reflect that the data suggest centromere-enrichment of Aurora B is sensitive to the *distribution* of

cohesin and ectopic transcription.

Page 8: Changes in centromere methylation correspond with sensitivity to Aurora B inhibition in cancer cells….
The title of this paragraph does not reflect the content. There is no evidence of changes in centromere methylation based on the mRNAseq data.

We have revised section subheadings, taking care not to implicate methylation changes when it is the methyltransferase/demethylase being assessed or manipulated.

Please indicate which phosphorylated residue is recognized by the pHec1 Ab. If it is S55, bear in mind that this site is not solely phosphorylated by Aurora B, but also by Aurora A. (DeLuca et al. 2017).

We have updated the text to indicate that the phosphorylation mark on Hec1 that we have monitored is S55. As the reviewer points out this residue can be phosphorylated by both Aurora B, and Aurora A. We have updated the text and figures to clarify this specific phosphor-residue, as well as others that are monitored in our studies. Consistent with a decrease in Hec1pS55, we see that cells expressing cen-Suv39 or cen-Suv420, as well as cancer cell lines that express these enzymes at a high level, exhibit increased sensitivity to both Aurora A and Aurora B kinases. These expanded analyses are represented in Figure 6 and the discussion section.

Lines 468-476: “We find centromere tethering of either Suv39 or Suv420 is sufficient to compromise Aurora B kinase localization at centromeres and result in reduced phosphorylation of Aurora B substrates that are critical for proper chromosomes segregation. Hec1, a key Aurora B substrate that governs kinetochore microtubule stability and mitotic error correction, is also a substrate for the related Aurora A kinase (DeLuca, 2017). Consistent with this redundant regulation, our data indicate that when centromere methylation is experimentally enhanced, or in cancer contexts where Suv39 or Suv420 expression is high and H4K20me3 is therefore likely high, sensitivity to inhibition of both Aurora A and Aurora B kinase is increased (Figure 6).”

There is no need to normalize the quantitative IF data (Fig 2 B-E; 4E-G, 5B-D, S2D, S3B, C, E). Showing all individual data points (also for the controls) is much more informative, since it will show the number of measurements per conditions as well as the spread in the control group. It could explain why the increase in H3T3ph and H2AT120ph after knockdown of KDM4A or expression of cen-Suv420 is quite large but not statistically significant (Fig. S3). Please indicate the statistical test used in figure legends. It would be very helpful to number the pages and figures.

We have replaced all data that reflect measurements from individual cell or kinetochores with superplots that show all measurements, color-coded by replicate. Our fluorescence data is represented as relative values (relative to the average measures in the control condition), but is otherwise not normalized (see also response to reviewer 1). We have revised the graph axes, figure legends, and methods sections to clarify this. We have added information about the number of measurements made for each experiment and the statistical tests used to each figure legend.

Please indicate the statistical test used in figure legends.
The figure legends have been updated to indicate the statistical test(s) used.

It would be very helpful to number the pages and figures. Pages and Figures have been numbered

Reviewer 3
Advance Summary and Potential Significance to Field:
It is known that histone methylation is commonly dysregulated in cancers. The manuscript by Herlihy et al describes that over-enrichment of histone H3K9me2/3 and/or H4K20me3 at the centromere inhibits Aurora B localization at the centromere and suppresses Aurora B dependent phosphorylation at the kinetochore, leading to a failure in error correction of kinetochore attachment and to chromosome missegregation. The authors manipulated these histone methylation marks by two methods; siRNA-mediated depletion of H3K9 demethylase KDM4A, and

artificial targeting of H3K9 methyltransferase Suv39 or H4K20 methyltransferase Suv420. They demonstrate that these treatments increase levels of H3K9me2 and H4K20me3, while decrease Aurora B level at centromeres and phosphorylation of known Aurora B targets, CENP-A and Hec1. These treatments also increased the mitotic chromosome defects such as lagging chromosomes, while depletion of KDM4A, demethylase inhibited mitotic error correction, the processes which are dependent on Aurora B. Since Suv420 is known to be recruited to H3K9me3 marks via HP1, and the effect of cen-Suv420-GFP on the Aurora B localization defect is much severer than cen-Suv39, the authors suggest that Suv420 acts downstream of Suv39 to inhibit Aurora B.
The impact of cen-Suv420-GFP expression on Aurora B localization looks interesting and surprising. Since this treatment did not affect known mechanism of Aurora B localization at the centromeres, such as H3T3ph and H2A T120ph, the study hints existence of a novel mechanism. While the results raise more questions than clarify existing questions, I find that these data have interesting implications in the future study. However, there are several concerns that must be clarified before publication.

We thank the reviewer for their thoughtful feedback and now include new data showing centromere transcript abundance is decreased when Suv39 or Suv420 is tethered to centromeres and propose that this change in transcript abundance may be relevant to the decrease in Aurora b localization at the centromere.

Reviewer 3 Comments for the Author:
Major points.

1. Page 4. “Global increases in H3K9me3 and H4K20me3 repressive heterochromatin marks have been described in several cancer contexts (Janssen et al., 2018, Black et al., 2012)”

These two cited review papers report several cases where these methylation processes are suppressed in cancers, but I could not find any specific description that these marks are globally increased in several cancers, as the authors imply. It is commonly known that transposons are activated in cancer cells, likely reflecting the decrease of these heterochromatic histone marks. Please cite original research papers that report the global increase in these heterochromatin marks in cancers.

We thank the reviewer for highlighting this lack of clarity in our introduction. As described above in response to reviewer 1 (who pointed out this same gap in logic) we now have clarified and expanded upon the introduction to emphasize that while many studies describe a relationship between a *decrease* in heterochromatin marks and tumorigenesis, other studies describe *increased* H3K9 and H4K20 methylation as promoting tumorigenicity. We have revised the introduction to better describe this apparent contradiction. We had previously cited a review by Janssen et al 2018 that discusses how decreased heterochromatin may compromise genome integrity, and prior work (Black, Manning et al. 2012) showing KDM4A loss/decreased expression in cancer (22.1% of tumors) is similarly prevalent to KDM4A gain/increased expression (18.9% of tumors). To better justify our studies and highlight prior work demonstrating a role for increased H3K9me3 and/or H4K20me3 in tumorigenesis, we now also include data from the TCGA showing that isoforms of both Suv420 and Suv39 expression is increased in many cancer contexts, compared to normal tissue, and cite work by Zhou et al., 2019 describing increased H3K9me3 and H4K20me3 in esophageal carcinoma, and work by Yokoyama et al, 2013 describing increased H3K9me3 in breast and colorectal cancer.

2. From method description, expression of cen-Suv39-GFP and cen-Suv420-GFP was done by transient transfection for some experiments and also by dox-induced expression of stably integrated gene in other experiments, but it is not clear which method was used for each specific experiment. This must be explicitly stated.

In the original manuscript only H3K9me2 and H4K20me3 staining of chromosomes spreads and Aurora B staining at centromeres was performed in cells transiently expressing the cen-Suv39 or cen-Suv420 constructs. However, in the course of revisions we have now repeated and replaced all experiments originally performed with the transient transfection of the cen-Suv39 or cen-Suv420 constructs with experiments performed in cell lines expressing doxycycline- regulated versions of these same expression constructs, save for the H3K9me2 staining. We have updated the methods and figure legends to more accurately describe the approach used to express

centromere-targeted fusions of Suv39 and Suv420.

Please specify which Suv39 and Suv420 isoforms (h1 or h2?) were used for centromere targeting.

The centromere-targeted fusions are of the Suv39h1 and Suv420h2 isoforms. We have updated the text to reflect this.

Since Suv39 and Suv420 accumulate at the inner centromeres and pericentromeres, the negative control of Suv39-GFP and Suv420-GFP must be done to show that the localization of cen-Suv39-GFP and cen-Suv420-GFP is dependent on CENP-B.

The reviewer correctly points out that both Suv39 and Suv420 normally localize to the pericentromeres and asks that we test whether merely overexpressing these enzymes (without the centromere tether) is sufficient to compromise mitotic fidelity, as we demonstrate with the cen-targeted constructs. As described above in response to a similar inquiry by Reviewer 1, we have now performed this experiment and shown that induced expression of Suv420-GFP in RPE-1 cells, in the absence of the centromere-targeted CENPB DNA binding domain tether does localize to chromatin, but is insufficient to promote enrichment of Suv420 at centromeres, increase centromere levels of H4K20me3, or alter Aurora B localization. This is consistent with our previously published working showing that overexpression of Suv420h2 alone in RPE cells does not significantly alter H4K20me3 levels at centromeres an pericentromeres (shown by ChIP, Manning et al., 2014). These data are now represented in Figure 3, and Supplemental Figure 3, and described in the text.

Lines 247-252: “Reduction in centromere localization of CPC components is dependent on centromere-enrichment of Suv420 as neither expression of centromere targeted GFP alone (cen-GFP) nor non-targeted Suv420 (Suv420-GFP) in RPE-1 cells is sufficient to both alter centromere H4K20me3 and disrupt Aurora B localization (Figure 3B, Figure 4B, and Supplemental Figure 3A).”

Also, in Figure S2E, compare the expression level of the fusion proteins with endogenous proteins. If the expression levels of the fusion proteins are massively higher than endogenous levels, it would be difficult to conclude that the observed phenotypes are caused by centromere targeting.

We now include western blots comparing the expression level of cen-Suv-GFP constructs to that of the endogenous proteins, the non-targeted Suv-GFP expression, as well as the expression of a centromere targeted GFP alone (cen-GFP). While each of our Suv expression constructs is expressed at levels much higher than the endogenous protein, we believe that this high expression alone (independent of centromere-targeting) can not explain the mitotic defects we describe as both the cen-GFP and the non-targeted Suv-GFP constructs are expressed at comparable or higher levels, yet do not compromise Aurora B localization. These new data are represented in Figure 3, Supplemental Figures 2 and 3, and detailed in the discussion

Lines 372-377: “… expression of centromere-targeted GFP, even at levels higher than that of cen-Suv39-GFP or cen-Suv420-GFP, is insufficient to alter centromere H4K20me3 or Aurora B localization (Figures 3B & 4B). Together, these data suggest that Aurora B localization and mitotic fidelity are not generally perturbed by protein tethering to the centromere but are instead specifically sensitive to Suv420 and/or H4K20me3 levels.”

3. Figure 2A. The data show that the increase of H4K20me3 is induced by targeting Suv39 (by cen-Suv39-GFP) or Suv420 (by cen-Suv420-GFP) to centromeres. However, in the case of cen-Suv39-GFP, there is a massive increase of H3K9me2 not only on centromeres but also on chromosome arms. The similar increase of H3K9me2 on chromosome arms can be seen with cen-Suv420-GFP. One of the common issues of IF staining with H3K9me2/3 staining is that the antibodies against these methyl marks do not react when the adjacent H3S10 is phosphorylated. If cen-Suv420-GFP indeed decreases Aurora B activity, this may increase H3K9me2 reactivity by decreasing H3S10ph levels. To resolve these confusions, it would be important to test if H3K9me2 antibody can be affected by H3S10ph, and also if cen-Suv420-GFP decreases H3S10ph levels. Authors should explain why this increase of methylation level at chromosome arms is induced.

To address the concern that changes in H3K9me2 staining may not accurately reflect changes in H3K9me2 levels we now include new experiments and additional analyses on the original IF experiments: We first assessed H3K9me2 and me3 levels in each condition with western blot analysis and by quantitatively measuring overall H3K9me3 that co-localizes with DAPI. As the reviewer inferred from the IF images, we do detect quantifiable changes in overall H3K9me2 and me3 staining (co-localized with chromatin) following cen-Suv39-GFP or cen-Suv420-GFP expression. However, western blot analyses of these same methylation marks show no change in mitotic populations of cells upon induction of cen-Suv39-GFP or cen-Suv420-GFP expression. As the reviewer proposes, and consistent with the changes we describe in Aurora B function, western blot analyses indicate that H3S10ph levels in nocodazole-arrested mitotic cells are decreased following both cen-Suv39-GFP and cen-Suv420-GFP expression. Together these data support the reviewer's hypothesis that a reduction of H3S10ph permits increased antibody access to H3K9me2, and explains the apparent non-centromere changes in this methyl mark as an artifact of the immunofluorescence assay. We thank the reviewer for these insightful suggestions to help better understand the changes in methylation patterns seen following expression of cen-Suv39-GFP and cen-Suv420-GFP. These new data are represented in Supplemental Figures 1 and 4.

In addition, chromosome architectures of some representative images look abnormal (cen Suv39 with H3K20me3 staining, cen-Suv420 with H3K9me2 staining). The method implies that the authors did not fix the samples at all for this analysis. The authors should provide rationale behind this procedure, and discuss whether the change in apparent chromosome morphology between samples could affect IF staining patterns.

To address this reviewer's concern, we have repeated the chromosome spreads for H4K20me3 in all conditions per the methods described in Martins et al., 2020. Although the chromatin arms are "crisper" with this new spread preparation, the results of the centromere analysis did not deviate from what was described in the original submission.

Statistical analyses do not appear to match with representative images.

We regret if any of the representative images did not reflect the statistical analyses. Though unsure to which analyses/representative images the reviewer is referring, we have reviewed each figure panel and carefully selected replacement panels, as needed, to ensure that the images are truly representative and reflective of the analysis.

4. Page 6, "Enhanced H4K20me3 compromises mitotic error correction mechanisms" and Figure 3, "Mitotic error correction is compromised when H3K9 methylation levels are increased". The experiments shown in Fig. 3 are solely based on KDM4A depletion by siRNA. Figure S2C showed that this treatment mildly increased H3K9me2 levels at centromeres, but never shown the effect on H4K20me3. The authors should monitored H3K9me2, H3K9me3, and H4K20me3 levels upon siKDM4A treatment by both IF and western. The authors should also change the titles for this section and the figure to better reflect the actual experiments.

We have updated figure and subheading titles to better reflect the data presented therein.

It is not clear if these experiments were performed with a pool of siRNAs or just one siRNA. It is a standard in the cell biology field that siRNA specificity is confirmed by expression of siRNA-resistant mutant. Reliance on the phenotypic analysis by a pooled siRNA is not acceptable.

Characterization of KDM4A depletion and anaphase defects were confirmed with each of 4 independent siRNA sequences. This data is reflected in Supplemental Figure 1. We have amended the text and methods to clarify this point and to better emphasize that the expression of cen-Suv39-GFP serves as a functional control for the specificity of altering the state of H3K9 methylation.

5. Figure 3C. Standard information behind statistics is missing. Chromosome misalignment can be caused by unstable kinetochore attachment or hyper-stable attachment. This can be assessed by cold resistance of microtubules and/or Mad2 staining.

We have updated all figure legends to include details of the statistical analyses and significance.

Per the reviewer's suggestion and to clarify whether the misalignment of chromosomes corresponds with increased or decreased kinetochore microtubule stability, we now include analysis of cold-stable microtubules following cen-Suv39-GFP or cen-Suv420-GFP expression. Consistent with our described decrease in Aurora B localization and function at centromeres, we find that expression of either cen-Suv39-GFP or cen-Suv420-GFP leads to an overall increase in MT stability, as indicated by the persistence of cold-stable microtubules in these conditions. This new data is represented in Figure 5 and described in the text.

Lines 254-259: "Phosphorylation of Hec1 (at serine 55) by Aurora B de-stabilizes kinetochore microtubule attachments to permit error correction. Consistent with compromised Aurora B localization, we find centromeres in mitotic cells have decreased Hec1 phosphorylation, but not overall levels of Hec1, when cen-Suv39-GFP or cen-Suv420-GFP is expressed (Figure 5A & B). These cells also exhibit a corresponding increase in stable microtubules that are resistant to cold-induced depolymerization (Figure 5C)"

6. Effect of cen-Suv420-GFP on Aurora B localization is striking. Since CPC formation is critical for Aurora B localization and activation, it would be important to check total expression levels and localization of the CPC subunits. It is also standard to examine if the CPC formation is affected by cen-Suv420-GFP using immunoprecipitation.

To address this concern we have confirmed that, similar to Aurora B, INCENP localization to the centromere is similarly disrupted by KDM4A depletion, or Suv39/Suv420 tethering to the centromere. We also have performed qPCR to assess Aurora B, INCENP, Borealin, and Survivin expression levels and find no difference in the presence or absence of induced expression of cen-Suv39-GFP or cen-Suv420-GFP. This data suggests that regulation of centromere localization, and not CPC levels *per se* are altered by centromere-tethering of Suv39 or Suv 420. These data are presented in Supplemental Figure 3, and discussed in the results section.

Although our attempts to immunoprecipitation the CPC, as suggested by the reviewer, were unsuccessful, we find that CPC complex localization to the centromere is only disrupted in contexts where Suv420 is tethered to centromeres via the CENPB DNA binding domain and not by expression of a non-targeted Suv420 construct. These data suggest that disruption of the CPC localization likely occurs at or proximal to the centromere, and may not perturb soluble CPC complex formation. We expand on this possibility in the discussion section.

7. The title "H4K20 methylation modulates Aurora B localization at centromeres and regulates mitotic fidelity" is not accurate. The authors show that Suv420 enrichment at the centromere interferes with Aurora B localization, but it remains to be tested if H4K20 methylation is important. It is possible that the critical target of Suv420 is not H4, and such a possibility should be discussed.

We have revised the title to "Suv420 enrichment at the centromere interferes with Aurora B localization and function" to better reflect the conclusions of our study.

Minor comments
1. Methods for fluorescent signal normalization are missing.

We have updated the methods to reflect that all fluorescence values have been normalized to the average of the values measured in the control condition and represented as fold-change in fluorescence.

2. 'Anaphase defects' are not well defined. An example shown in Figure1H do not show the typical lagging chromosome phenotype. As an alternative measure for chromosome segregation defect, frequency of micronuclei would be informative.

While we do see a range in the degree to which chromosomes lag in the KDM4A depletion condition, most cells exhibit a more obvious lagging chromosome phenotype and we regret that the chosen image that does not clearly reflect this. We have replaced the image in Figure 1H with

one that better reflects the phenotype we have scored (now Figure 2C), and clarified in the methods that we define lagging chromosomes as those that meet the following criteria: are located between the two masses of anaphase chromosomes (anaphase plates) and at least as far from the next nearest kinetochore as the anaphase plate is wide (based on ACA staining).

Per the reviewer's suggestion, we have also now quantified the frequency of micronuclei present in populations of cen-Suv39 and cen-Suv420-expressing cells. Consistent with the observed increase in lagging chromosomes, we find that populations of cells expressing cen-Suv39-GFP or cen-Suv420-GFP also display in increase in the number of micronuclei present. This new data is represented in Figure 3C and described in the text.

Lines 186-189: "Consistent with the presence of mitotic segregation errors, cells expressing cen-Suv39-GFP or cen-Suv420-GFP also exhibit an increase in the number of interphase cells within the population that have centromere-positive micronuclei (Figure 3C)."

3. In this paper, all statistical analyses are done by Student's two tailed t-test. Students' t-test has to be applied only to the 'normally distributed' data sets. It would be important to show data distribution, and justify the usage of this statistical analysis. Also, describe the number of analyzed samples/experiments for each panel.

We have updated all graphs depicting single cell and single-kinetochore measurements to super-plots that better display the distribution of measurements within each sample and between replicates. We have also updated the methods and all figure legends to include details of the number of samples measure per replicate, the number of replicates, as well as a description of the statistical analyses applied for each type of experiment.

4. Page 14. H3K9me3 must read H3K9me2.

Much of the data in the original submission to assess H2K9me2 has now been supplemented with analysis of H3K9me3. We have revised the text to ensure that "H3K9me2" and "H3K9me3" are used as appropriate.

5. Hec1 has multiple phosphorylation sites. Please specify which phosphorylation site was monitored. The pHec1 antibody apparently stains at the regions devoid of chromosomes, and not specifically at the kinetochores in Mock sample. How do you know the specificity of the antibody?

The pHec1 antibody we used detects pS55. We have updated the text and figures to reflect this. Hec1 has been described to localize both the kinetochores, and to centrosomes (Hori *et al.*, 2003 ; Sauer *et al.*, 2005 ; Lin *et al.*, 2006 ; Goshima *et al.*, 2007 ; Diaz-Rodriguez *et al.*, 2008) and this antibody detects both pools. Validation by Thermofisher (PA5-85846) demonstrates that both kinetochore and centrosome localized staining with this antibody is eliminated following siRNA-based depletion of Hec1.

In Figure 5C, pHec1 signals are massively accumulated at the centrosome when they expressed cen-Suv420-GFP compare to Mock. Is this reproducible?

Our focus on preparing these images was on the kinetochore pool of Hec1 and we neglected to notice that the spindle poles in this particular cell are out of focus with the central plane of kinetochores and hence are not visible in this cropped image. We have confirmed that a decrease in centrosome pHec1 is not apparent in the full volume image of this cell, or any other cell examined that is expressing cen-Suv420-GFP. We thank the reviewer for bringing this to our attention and have now replaced the representative image of the 'mock' condition with one in which both spindle poles remain within the focal plane.

6. Figure 2A. Mock treatment images on upper right panel look fuzzier than other images. Please confirm that those images were correctly deconvoluted.

To address this and other concerns raised by multiple reviewers, we have repeated all chromosome spreads and stained for H4K20me3 using a revised protocol. Though the images are

crisper, the analysis remains consistent with what was described for the original images.

7. Figure 4B-D. Define AFUs, and what does the value 1.0 means. The AFU values in B-D do not match to AUC AFUs shown in E-G. What does AUC AFU mean here, and how were the values calculated? Also, define it in Figure 2B-E.

The graph axes, figure legends, and methods have all been revised to reflect that measurements are represented as fold-change in fluorescence (previously inaccurately referred to as arbitrary fluorescence units, or AFU)

8. Figure 5E. Please define IC50 AUC.

We thank the reviewer for pointing out this error. The Sanger drug sensitivity data is presented as EITHER IC50, or AUC. The values used in our analyses were AUC values. AUC is defined by the Sanger Institute as the area under the fitted dose response curve. This value summarizes the dose response curve to a single number that corresponds to the degree of sensitivity for each cell line to a given compound. We have updated the text and methods to reflect this.

9. Please specify the version of R programming environment and plugin for statistical analysis used in patient survival data analysis.

The R programming version 3.3.1 was used for these analyses. The Kaplan-Meier Plotter platform (kmplot.com/analysis; (Nagy et al., 2018, Győrffy et al., 2010)) was used to query survival data relative to Suv39 and Suv420 isoform expression with automatic cutoffs and without further restrictions imposed. Bonferroni-corrected p values where determined and significance defined as p<= 0.0053.

10. Add an abbreviation for HR in Figure 1A-C and S1as hazard ratio.

These analyses have been moved to Supplemental Table 2.

Second decision letter

MS ID#: JOCES/2020/249763

MS TITLE: Suv420 enrichment at the centromere interferes with Aurora B localization and function

AUTHORS: Conor P Herlihy, Sabine Hahn, Nicole M Hermance, and Amity Manning
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

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

As you will see, the reviewers gave favourable reports but raised some critical points that will require amendments to your manuscript. I hope that you will be able to carry these out, because I would like to be able to accept your paper.

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

provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

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

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

Reviewer 1

Advance summary and potential significance to field

N/A

Comments for the author

The paper has been significantly improved by the revisions, with a more robust analysis of the correlation of Suv39 and Suv420 isoform expression in multiple cancers with aneuploidy and tumour progression, as well as a more complete analysis of the inhibitor data in Figure 6. Although the mechanisms of the effects of cen-Suv39 and cen-Suv420 remain unclear, there is now at least a pointer to the idea that changes in centromeric transcription are involved. In general, the chromosome segregation defects seem more pronounced than the changes in histone methylation. This does question whether the mechanism involves histone methylation at all, but this is acknowledged in the discussion.

I do think some additional points need to be addressed prior to publication.
1. What looks like log10 and log2 scales are now used on many graphs though this is not stated. Also the numbers on the scales shown are inconsistent. Personally, I find this confusing. A linear scale would be easier to interpret for these small changes, and would give a more intuitive view of the magnitudes. Also the choice of colours for the mean boxes on the graphs make them difficult to see.

2. In the previous version of the paper, it seemed clear that histone methylation was increased on chromosome arms in some instances. This is not so clear in the revised version. Does cen-Suv expression alter arm methylation or not?

3. In supplemental figure 4c, am I correct in thinking that H3S10ph disappears when cen-Suv proteins are induced (the legend does not make fully clear what "+" means)? This is very surprising given the small changes in histone methylation and phosphorylation observed by IF. Why the discrepancy?

4. Related to points 3 and 4, reviewer 3 brought up the good point that H3K9me antibodies can be blocked by H3S10ph (and, indeed, vice versa). However, each antibody is different, and has to be tested individually. As mentioned by reviewer 3, the specific antibodies used here would have to be tested to determine if epitope blocking is an issue. I can say that our own data show that anti-H3S10ph antibody 6G3 (CST 9706) is strongly blocked by H3K9me3. The authors do not appear to have directly addressed this point, and it is relevant to both IF and immunoblotting approaches. Does H3 methylation (or H3S10ph)

only appear to increase (or decrease) because of antibody epitope issues? This is an important point because it questions whether there are real changes in the extent of both of these histone modifications not only on chromosome arms but also at centromeres.

5. In supplemental figure 1E, it is not clear what is being measured in the graphs in the 2nd vs 3rd columns.

6. In figure 4B, how do we know that the cells analysed express the GFP constructs? This is particularly relevant for cen-GFP that was expressed transiently, and so likely in a minority of cells.

7. In figure 5c, the mock cells seem to be late prometaphase, while the induced cells are metaphases.
This is opposite to the reported predominant phenotype (ie alignment problems in induced cells), and will potentially favour seeing stable microtubules in the metaphases. Has this been accounted for, for example by doing the analyses in a blinded fashion?

8. In figure 6, there seem to be clear increases in sensitivity to all inhibitors tested, and it is unclear what is being compared to produce the significance asterisks. Overall, there is a danger that these data are being selectively interpreted, and a clearer statistical analysis is needed. In addition, bar charts are not an optimal way to display these kinds of dose-response data (why not line graphs?).

9. In supplemental figure 5D, it would be useful to have a non-centromeric transcript analysed. Without something analogous to a loading control, how do we know that the "induced" samples are not simply under-represented, or that there is not a global reduction in transcription?

10. The results in supplemental figure 6 are really not compelling. It makes little sense to me to say that there is a correlation "in some cases" (line 367). A more robust analysis of the data is required here. Is there a correlation, or is there not?

11. Finally, the Discussion could perhaps be tightened up to give a more coherent view of the authors'
preferred model, while still acknowledging alternative interpretations.

Reviewer 2

Advance summary and potential significance to field

Herlihy et al describe how centromere enrichment of Suv420 and a concomitant increase in H4K20Me3 results in a reduction of Aurora B levels and function. While the data that show this is convincing the physiological relevance of these findings remain very limited, mainly due to the experimental setup, which relies on artificial tethering of Suv420 to centromeres.

Comments for the author

The authors continually fall back on correlations identified in databases as a support or justification for their work. However, increasing centromere levels of the described methylation marks through forced expression of CENP-B-suv39 or CENP-B-suv420 in no way compares to global increases as observed in some cancers. It is not surprising that forcing a chromatin modifying enzyme to centromeres will result in mitotic defects! The use of CENP-B fusions here is different than the majority of cases where CENP-B is typically used to put back a certain activity to rescue a phenotype, thereby providing some evidence for causality. Importantly, the authors show that global overexpression of suv420 (a situation, according to the authors more mimicking certain cancers) leads to both global increases in H4K20me3 as well as at centromeres but does NOT reduce Aurora B levels at centromeres. Moreover, in a panel of breast cancer cells with varying levels of centromere H4K20Me3 there is no clear correlation with Aurora B levels. But even there, regardless of the outcome, the data only shows correlations and does not prove causality.
If the authors could show experimentally that increased expression of Suv39 or Suv420 (not centromere targeted!) results, in some context, in a phenotype resembling the database correlations they draw from they would have a solid basis for an interesting manuscript. To prove causality, they would need to then reduce H4K20Me3 and show this increases Aurora B levels and rescues chromosome alignment and missegregation phenotypes. Alternatively, they could/should show that restoring Aurora B levels by another means (CENP-B INCENP) rescues chromosome alignment phenotypes in the presence of high Suv420/H4K20Me3. Importantly, rescuing or restoring Suv420/H4K20Me3 or Aurora B levels would show the causality required to draw the conclusions the authors are trying to make. I cannot support publication of this manuscript.

Reviewer 3

Advance summary and potential significance to field

In this revised version, the authors made great efforts to address my original concerns with a number of new data, which significantly improved the overall impact of this study. The study clearly demonstrates that forced centromere enrichment of histone methyltransferases Suv39 and Suv420 suppressed centromere recruitment and function of Aurora B and caused chromosome missegregation.
Although mechanistic basis of this phenomenon is not clear, potential implications in cancers with high expression of Suv420 and Aurora inhibitors are interesting.

Comments for the author

I recommend publication of the revised version after dealing with a few minor issues.
1. I highly recommend the authors rewrite the abstract. The current version of the abstract spends a majority of space in implication of this study in cancers, but the data in this point are rather correlational. The main experiments of the paper are the effect of KDM4A depletion and centromere targeting of SUV39 and SUV420. In the abstract, there is only one sentence refers to this major point; “Our data show that increased methylation of H3K9 and H4K20 compromises centromere recruitment of the mitotic Aurora B kinase and corrupts mitotic chromosome segregation.” As I stated in my original review, the experiments did not “show” that increased methylation of H3K9 and H4K20 caused the observed mitotic failure. Instead, the procedures to enhance these modifications did that.
Importantly, knockdown of KDM4A did not significantly increase H4K20me3 on the centromere, yet it decreased Aurora B levels on the centromere. In addition, it seems that cen-Suv420 expression increased H3K9 methylation (Supplementary Figure S1). Therefore, it is not clear if the observed defect in Aurora B localization and function is mediated through H4K20me3.
Since the results of cen-Suv39 and cen-Suv420 are robust and compelling, I feel strongly that the abstract should highlight these experiments.

2. Supplemental Fig. 6. It would be important to show Aurora B immunofluorescence images. There is a general tendency that reduced Aurora B enrichment on the centromere causes enhanced Aurora B on chromosome arms, so I’m curious to know if this is the case in these cancer cell lines.

Second revision

Author response to reviewers' comments

We are grateful to the helpful critique of our manuscript provided by each reviewer, and for the opportunity to revise and resubmit our manuscript. In response to the comments provided we have addressed each of the reviewer’s remaining concerns through clarifications in the text and figures, additional analysis, and new experimentation. We now include critical rescue experiments to demonstrate a casual relationship between Suv420/H4K20me3 at centromeres and Aurora B disruption. Following is a point-by-point response to each concern raised.

Response to reviewer comments

Reviewer 1 Comments for the Author:
The paper has been significantly improved by the revisions, with a more robust analysis of the correlation of Suv39 and Suv420 isoform expression in multiple cancers with aneuploidy and tumour progression, as well as a more complete analysis of the inhibitor data in Figure 6. Although the mechanisms of the effects of cen-Suv39 and cen-Suv420 remain unclear, there is now at least a pointer to the idea that changes in centromeric transcription are involved. In

general, the chromosome segregation defects seem more pronounced than the changes in histone methylation. This does question whether the mechanism involves histone methylation at all, but this is acknowledged in the discussion.I do think some additional points need to be addressed prior to publication.

1. What looks like log10 and log2 scales are now used on many graphs though this is not stated. Also, the numbers on the scales shown are inconsistent. Personally, I find this confusing. A linear scale would be easier to interpret for these small changes, and would give a more intuitive view of the magnitudes. Also, the choice of colours for the mean boxes on the graphs make them difficult to see.

We have replaced all graphs to plots with a new color combo to make the mean boxes more apparent. Axes for all single cell data are now linear with conserved scale for a given assay across all figures.

2. In the previous version of the paper, it seemed clear that histone methylation was increased on chromosome arms in some instances. This is not so clear in the revised version. Does cen-Suv expression alter arm methylation or not?

We find that cen-Suv expression leads to a small but significant increase in overall H9K9me3 and no increase in H4K20me3 levels. These measurements are now reflected in Supplemental Figures 2B and 1E. Importantly, due to a lack of chromosome arm-specific marker with which to assess co-localization, these measures assess all DAPI- associated H9K9me3, including that at centromeres and telomeres and is not exclusive to chromosome arms.

3. In supplemental figure 4c, am I correct in thinking that H3S10ph disappears when cen-Suv proteins are induced (the legend does not make fully clear what "+" means)? This is very surprising given the small changes in histone methylation and phosphorylation observed by IF. Why the discrepancy?

We thank the reviewer for highlighting this discrepancy. To clarify the relevance of the changes seen in the western blot analysis, we revised our cell synchronization approach and used a different H3S10ph antibody. The initial western blot analyses were performed on cells following an extended mitotic arrest. We now know that in cen-Suv expressing cells, an extended mitotic arrest permits increased/spreading methylation beyond the centromere-localized changes seen in other experiments (all of which were assessed during normal mitotic duration or following 1-3h of mitotic arrest). That levels of this phosphorylation mark decrease concurrent with spreading of H3K9me3 indicate that, as this reviewer suggests, our original analyses were biased by epitope masking that limited detection by the 6G3 monoclonal anti-H3S10ph antibody we had used. To eliminate the mitotic duration variable and also address reviewers' concerns, we have repeated these experiments using a short term nocodazole treatment and shake off approach to enrich for mitotic cells. We also now use an antibody that had been previously validated to recognize H3S10ph, irrespective of H3K9me3 status (Rothbart et al., 2015). This modified approach demonstrates only a moderate reduction in H3S10ph following cen- Suv39 expression and no change following cen-Suv420 expression. We have replaced the original panels C and D in supplemental figure 4 (now Supplemental Figure 5) with this new data and also include discussion relating this lack of change in H3S10ph to that seen with other perturbations that specifically perturb centromere localization of AurB (like Haspin depletion: Wang et al., 2010).

4. Related to points 3 and 4, reviewer 3 brought up the good point that H3K9me antibodies can be blocked by H3S10ph (and, indeed, vice versa). However, each antibody is different, and has to be tested individually. As mentioned by reviewer 3, the specific antibodies used here would have to be tested to determine if epitope blocking is an issue. I can say that our own data show that anti-H3S10ph antibody 6G3 (CST 9706) is strongly blocked by H3K9me3. The authors do not appear to have directly addressed this point, and it is relevant to both IF and immunoblotting approaches. Does H3 methylation (or H3S10ph) only appear to increase (or decrease) because of antibody epitope issues? This is an important point, because it questions whether there are real changes in the extent of both of these histone modifications, not only on chromosome arms but also at centromeres.

We thank the reviewer for stressing this point. As described in response to point 3, use of an antibody that had been previously validated to recognize H3s10ph, irrespective of H3K9me3 status does indeed show that this phospho mark is not dramatically altered by centromere tethering of cen-Suv constructs. New analysis of H3K9me3 and H4K20me3 levels along chromosome arms similarly supports a view that tethering of Suv-39 or Suv-420 to centromeres enhances methylation at centromeres, with little impact on bulk methylation levels as detected by immunofluorescence (on arms+ centromeres; Supplemental Figures 2B & 1E: see also response to pt 2 above). Supplemental Figures 2 and 4, and corresponding text has been updated to reflect this analysis.

5. In supplemental figure 1E, it is not clear what is being measured in the graphs in the 2nd vs 3rd columns.

We have updated these labels in this figure and the text in the corresponding legend to clarify that graphs in the left column depict measurements made at the centromere and graphs in the right column depict measurements made along all DAPI-stained chromatin.

6. In figure 4B, how do we know that the cells analysed express the GFP constructs? This is particularly relevant for cen-GFP that was expressed transiently, and so likely in a minority of cells.

Although not shown, all cells analyzed were selected based on being GFP+. To emphasize this, we now indicate this validation step in our methods and have now included the GFP panels for each representative image.

7. In figure 5c, the mock cells seem to be late prometaphase, while the induced cells are metaphases. This is opposite to the reported predominant phenotype (ie alignment problems in induced cells), and will potentially favour seeing stable microtubules in the metaphases. Has this been accounted for, for example by doing the analyses in a blinded fashion?

The cells imaged and measured were all selected based on chromosome alignment and all populations analyzed show similar distributions of what the review suggests may be late prometaphase and metaphase. Nevertheless, to mitigate the potential for bias in selection of cells to image, the analysis was blinded and repeated by a lab member. This additional replicate was found to be consistent with what we originally reported. To avoid misrepresenting the data, we have replaced the representative images to better reflect the similar mitotic staging across conditions.

8. In figure 6, there seem to be clear increases in sensitivity to all inhibitors tested, and it is unclear what is being compared to produce the significance asterisks. Overall, there is a danger that these data are being selectively interpreted, and a clearer statistical analysis is needed. In addition, bar charts are not an optimal way to display these kinds of dose-response data (why not line graphs?).

We have replaced the original bar graphs with line graphs depicting the sensitivity of each cell line (with and without induction of its respective cen-Suv-GFP construct) to Aurora kinase or MPS1 inhibition. We have performed an ANOVA analysis to compare the slopes of cell line each pair (mock vs induction) and find that induction of cen-Suv39 or cen-Suv420 renders cells more sensitive (ie steeper slope) to Aurora Kinase inhibition but not to MPS1 inhibition (cen-Suv39: p= 0.00003, 0.0003, and 0.844 for alisertib, barasertib, and MPS1IN1, respectively; cen-Suv420: p = 0.003, 0.004, and 0.724 for alisertib, barasertib, and MPS1IN1, respectively).

9. In supplemental figure 5D, it would be useful to have a non-centromeric transcript analysed. Without something analogous to a loading control, how do we know that the “induced” samples are not simply under-represented, or that there is not a global reduction in transcription?

All qPCR analysis is analyzed per the double delta Ct method that considers transcript changes relative to an internal control that is insensitive to the experimental manipulations (in this case GAPDH transcript). Additional detail has been added to the methods to reflect this. These same

conditions have also been analyzed for transcript levels of CPC components (Aurora B, Borealin, INCENP, and Survivin) and show no reduction (Supplemental Figure 3C), further confirming that reduction in centromere transcripts is not an indication of a global reduction in transcription. We now highlight these controls in the text and figure legend.

10. The results in supplemental figure 6 are really not compelling. It makes little sense to me to say that there is a correlation "in some cases" (line 367). A more robust analysis of the data is required here. Is there a correlation, or is there not?

We agree with this reviewer that our analysis of the breast cancer cell lines was limited by sample size as well as the genetic complexity of the cell lines analyzed. As we were unable to obtain additional cell lines with which to explore the relationship between Suv420 expression and centromere levels of Aurora B, we have removed these data.

11. Finally, the Discussion could perhaps be tightened up to give a more coherent view of the authors' preferred model, while still acknowledging alternative interpretations.

We have revised the discussion to clarify that we believe the mitotic defects we describe are unlikely to result from gross corruption of the centromere/kinetochore as key components of each (CENPA and Hec1) continue to localize properly and chromosomes continue to form end-on microtubule attachments that are competent to drive chromosome movement. We also note that the lack of defects in our cen-GFP expressing controls suggests that merely tethering a protein to the centromere is insufficient to similarly corrupt mitotic fidelity. We have also now expanded discussion on our view that changes in centromere transcription may be integral to the mitotic defects that result from tethering Suv420 to centromeres.

Reviewer 2 Advance Summary and Potential Significance to Field:
Herlihy et al describe how centromere enrichment of Suv420 and a concomitant increase in H4K20Me3 results in a reduction of Aurora B levels and function. While the data that show this is convincing the physiological relevance of these findings remain very limited, mainly due to the experimental setup, which relies on artificial tethering of Suv420 to centromeres.

Reviewer 2 Comments for the Author:
The authors continually fall back on correlations identified in databases as a support or justification for their work. However, increasing centromere levels of the described methylation marks through forced expression of CENP-B-suv39 or CENP-B-suv420 in no way compares to global increases as observed in some cancers.

It was not our intent to imply that global overexpression of suv420 (vs centromere enrichment) more closely mimics what is seen in cancer cells. Indeed, large scale analysis of whether protein overexpression of Suv420 (or really any transcript encoding for chromatin modifying enzymes shown to be increased in cancer contexts) is similar/dissimilar to enhanced localization locally or globally is lacking.

To clarify this point we have now performed additional analysis on our experimental RPE cell lines and a panel of breast cancer cells to assess the enrichment of H4K20me3 at centromeres (calculated as the average intensity of H4K20me3 staining at ACA-labeled centromeres relative to the average intensity of H4K20me3 staining at DAPI-stained chromatin of the same cell). As expected, we find that our cen-tethered constructs enhance the centromere enrichment of H4K20me3 beyond that seen in control cells. Our cen-GFP construct does not alter the centromere enrichment score and our untethered Suv420-GFP construct enhances overall H4K20me3, resulting in a decreased centromere enrichment score. Similar analysis of our panel of breast cancer cell lines, characterized by TCGA analysis as having high Suv420 expression, indicates that each of these cell lines exhibits centromere enrichment of H4K20me3, consistent with what we see with our cen-tethered Suv constructs and distinct from what we achieve with overexpression of untethered Suv420. Given that increased expression of the Suv420 enzyme alone is insufficient to drive its centromere enrichment in our experimental system, these data suggest that our centromere-tethering approach does indeed compare favorably (at least at the level of centromere enrichment of H4K20me3) to what is observed in cancer cells that express Suv420

highly. To illustrate the distribution of H4K20me3 in cancer cells with high Suv420 expression we now include measures of H4K20me3 centromere enrichment analysis in the our panel of breast cancer cell lines (Supplemental Figure 1) and expand the discussion to comment on how this distribution supports the relevance of our centromere tethering system.

It is not surprising that forcing a chromatin modifying enzyme to centromeres will result in mitotic defects! The use of CENP-B fusions here is different than the majority of cases where CENP-B is typically used to put back a certain activity to rescue a phenotype, thereby providing some evidence for causality. Importantly, the authors show that global overexpression of suv420 (a situation, according to the authors more mimicking certain cancers) leads to both global increases in H4K20me3 as well as at centromeres but does NOT reduce Aurora B levels at centromeres. Moreover, in a panel of breast cancer cells with varying levels of centromere H4K20Me3 there is no clear correlation with Aurora B levels. But even there, regardless of the outcome, the data only shows correlations and does not prove causality.

Contrary to this reviewer's assertion that only global overexpression of Suv420 could model a cancer context with high expression of Suv420, we find that patterns of H4K20me3 in a panel of breast cancer cells with high expression of Suv420 to be more similar to that seen in our centromere-tethered Suv420 model (enriched at centromeres) then in cells with overexpression of untethered Suv420 (increased along arms with a corresponding decrease in centromere enrichment). We propose in the discussion that this contradiction may reflect regulatory pathways present in normal cells but lacking in cancer contexts that function to limit Suv420 enrichment or otherwise restrict H4K20me3 at centromeres.

If the authors could show experimentally that increased expression of Suv39 or Suv420 (not centromere targeted!) results, in some context, in a phenotype resembling the database correlations they draw from, they would have a solid basis for an interesting manuscript.

As described above, the H4K20me3 staining patterns seen following centromere tethering of Suv420 do indeed reflect what is seen in cancer contexts.

To prove causality, they would need to then reduce H4K20Me3 and show this increases Aurora B levels and rescues chromosome alignment and missegregation phenotypes. Alternatively, they could/should show that restoring Aurora B levels by another means (CENP-B INCENP) rescues chromosome alignment phenotypes in the presence of high Suv420/H4K20Me3.

To test the causal relationship between Suv420/H4K20me3 enrichment and Aurora B regulation at centromeres, we now quantify Aurora B staining intensity at centromeres and chromosome alignment in cen-Suv420-GFP expressing cells in the presence and absence of the Suv420-specific inhibitor A196 (Bromberg et al., 2017). We find that treatment with A196 reduced H4K20me3 and promotes a partial rescue in Aurora B localization to centromeres. Consistent with restored Aurora B localization and activity, in cen-Suv420-GFP cells that are treated with A196 alignment of chromosomes at the metaphase plate is tighter. These new data are represented in Figure 5.

To test whether changes in Aurora B are correlative or causative to the mitotic segregation errors observed when cen-Suv420-GFP is expressed, we co-expressed cen-INCENP-mCherry and monitored for frequency of anaphase lagging chromosomes. We find that following transfection of cen-INCENP- mCherry, anaphase defects are reduced by half in cen-Suv420-GFP expressing cells. This reduction is not simply due to competition between cen-Suv420 and cen-INCENP for binding to the CENPB box as the localization of cen-Suv420-GFP is not statistically different in the presence of absence of cen- INCENP-RFP. These new data are represented in Figure 7.

Importantly, rescuing or restoring Suv420/H4K20Me3 or Aurora B levels would show the causality required to draw the conclusions the authors are trying to make.
I cannot support publication of this manuscript.

As described above, we now show that reduction of H4K20me3 using the Suv420-specific inhibitor A196 is sufficient to mitigate loss of Aurora B at centromeres and promote chromosome alignment during metaphase. We also now show that expression of cen-INCENP-mCherry in cells with

enriched Suv420/H4K20me3 at centromeres suppresses mitotic defects. Together with data included in previous submissions showing that the reduction in Aurora B localization is apparent in contexts where Suv420/H4K20me3 is enriched at centromeres and not following expression of cen-GFP or non- tethered Suv420-GFP (which do not result in centromere enrichment of H4K20me3) this supports a causal relationship between Suv420/H4K20me3 enrichment and negative regulation of Aurora B localization and activity at centromeres.

Reviewer 3 Advance Summary and Potential Significance to Field:
In this revised version, the authors made great efforts to address my original concerns with a number of new data, which significantly improved the overall impact of this study. The study clearly demonstrates that forced centromere
enrichment of histone methyltransferases Suv39 and Suv420 suppressed centromere recruitment and function of Aurora B and caused chromosome missegregation.
Although mechanistic basis of this phenomenon is not clear, potential implications in cancers with high expression of Suv420 and Aurora inhibitors are interesting.

Reviewer 3 Comments for the Author:
I recommend publication of the revised version after dealing with a
few minor issues.

1. I highly recommend the authors rewrite the abstract. The current version of the abstract spends a majority of space in implication of this study in cancers, but the data in this point are rather correlational. The main experiments of the paper are the effect of KDM4A depletion and centromere targeting of SUV39 and SUV420. In the abstract, there is only one sentence refers to this major point; “Our data show that increased methylation of H3K9 and H4K20 compromises centromere recruitment of the mitotic Aurora B kinase and corrupts mitotic chromosome segregation.” As I stated in my original review, the experiments did not “show” that increased methylation of H3K9 and H4K20 caused the observed mitotic failure. Instead, the procedures to enhance these modifications did that.

We have reworked the abstract to better reflect the data presented in this manuscript Importantly, knockdown of KDM4A did not significantly increase H4K20me3 on the centromere, yet it decreased Aurora B levels on the centromere. In addition, it seems that cen-Suv420 expression increased H3K9 methylation (Supplementary Figure S1). Therefore, it is not clear if the observed defect in Aurora B localization and function is mediated through H4K20me3.

Unlike expression of the cen-targeted constructs whose GFP tag allows us to confirm that each mitotic cell/centromere analyzed has overexpressed the enzyme, we do not have a similar report of KDM4A depletion at the single cell level and can not rule out the possibility that changes in methylation/AurB staining at centromeres may be impacted by variability of KDM4a depletion with the cell population.
Further complicating the interpretation of these data, our fixation conditions for H4K20me3 are incompatible with those for Aurora B staining, precluding us from making a direct correlation between H4K20me3 and Aurora B at the single cell/single centromere level. Nevertheless, new data showing that inhibition of Suv420 methyltransferase activity (with A196) only partially restores Aurora B localization is consistent with Aurora B localization being at least partially independent of H4K20me3 levels. We now introduce these limitations in the discussion and raise the possibility that Aurora B localization may be independently influenced by H3K9me3, H4K20me3 and/or the localization of the enzymes that place these marks.

Since the results of cen-Suv39 and cen-Suv420 are robust and compelling, I feel strongly that the abstract should highlight these experiments.

We have revised the abstract to better highlight the experiments performed with cen-Suv39 and cen- Suv20 expression.

2. Supplemental Fig. 6. It would be important to show Aurora B immunofluorescence images. There is a general tendency that reduced Aurora B enrichment on the centromere causes enhanced Aurora B on chromosome arms, so I’m curious to know if this is the case in these cancer cell lines.

Unfortunately our analysis of the breast cancer cell lines was limited by sample size as well as the genetic complexity of the cell lines analyzed. As we were unable to obtain additional cell lines with which to explore the relationship between Suv420 expression and centromere levels of Aurora B, we have removed these data. Though this reviewer may be interested to know that in all five breast cancer cell lines analyzed, regardless of absolute Aurora B staining intensity, Aurora B distribution remained enriched at centromeres (ie we did not see evidence of dramatic redistribution to chromosome arms).

Third decision letter

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AUTHORS: Conor P Herlihy, Sabine Hahn, Nicole M Hermance, Elizabeth A Crowley, and Amity Manning

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I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.