Chromosome oscillation promotes Aurora A-dependent Hec1 phosphorylation and mitotic fidelity

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Re: JCB manuscript #202006116

Prof. Kozo Tanaka
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Dear Prof. Tanaka,

Thank you for submitting your manuscript entitled "Chromosome oscillation promotes Aurora A-dependent Hec1 phosphorylation and mitotic fidelity". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. As you can see, two reviewers are more enthusiastic about the study than the third reviewer, but all raised several important concerns. We find the work important and interesting but agree with these concerns. Therefore, we would like to invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

1. As Reviewer #3 points out, it is critical to validate the specificity of anti-phospho-Hec1 Ser55 by siRNA/rescue and peptide competition.

2. Reviewer #3 pointed out that HCT116 is a non-CIN cancer cell line, and thus dampened chromosome oscillation may not relate to chromosome missegregation. Dr. Duane Compton and colleagues have suggested that CIN-cancer cells have stable kinetochore microtubules, leading to chromosome missegregation. It is important to relate your results with the Compton observations and further explain this issue.

3. Reviewers #1 and #2 point out the possible effect of MG132 on chromosome oscillation. This concern must be clarified by additional control experiments.

4. Reviewers #1 and #2 point out that the Aurora A S155R mutation may not just affect spindle localization but also inactivate the kinase activity. An additional better separation-of-function mutant, suggested by Reviewer #1, must be tested.

5. Reviewer #3 questions the conclusion that your major kinase for Hec1 S55 phosphorylation is Aurora A. Since this conclusion stands in contrast with the previous observations by Dr. Jennifer Deluca and colleagues, it is essential to clarify and address this point.

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

GENERAL GUIDELINES:

Text limits: Character count for an Article is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does
Reviewer #1 (Comments to the Authors (Required)):

Chromosome oscillations are a well-known phenomenon in mitosis, but their function has long been unclear. This manuscript links oscillations to chromosome segregation fidelity. The data show lower amplitude oscillations and reduced phosphorylation of Hec1-S55 (an Aurora A substrate) in
transformed cells compared to untransformed cells. Furthermore, oscillation amplitude and Hec1 phosphorylation depend on each other, with manipulations of one affecting the other. Treatments that increase oscillation amplitude also reduce lagging chromosomes that result from merotelic attachment errors, whereas Aurora A inhibition or mutations that prevent Hec1 phosphorylation increase lagging chromosomes. Together, these findings support a role for oscillations in resolving merotelic chromosomes by promoting Hec1 phosphorylation. Overall, this work represents a significant advance by providing a function for chromosome oscillations in regulating kinetochore-microtubule interactions.

Major comments:

1. The overall model for how oscillations eliminate incorrect attachments is unclear. First, how does Hec1 phosphorylation promote error correction? Is it by increased microtubule turnover at kinetochores? If so, this should be stated explicitly? Second, is there any selectivity for merotelic attachments? Is the oscillation amplitude larger for merotelics, which would bring them closer to spindle poles? Alternatively, if oscillations affect all kinetochores equally, then the authors should explain how this facilitates error correction.

2. The interpretation of the Aurora A S155R experiment is unclear because TPX2 is an Aurora A activator, and thus the mutation is expected to affect kinase activity as well as microtubule binding. Microtubule binding and kinase activity could potentially be decoupled using TPX2 mutants characterized by Zhang et al. (2017 eLife, PMID 29120325). Otherwise, the conclusion that spindle-localized Aurora A is responsible for Hec1-S55 phosphorylation is not well supported.

3. For the Aurora A inhibition or depletion experiments in Fig. 6C, it is important to show that the spindle is not disrupted. Otherwise lagging chromosomes might be due to more general spindle problems rather the authors' interpretation.

Minor comments:

1. Using MG132 might confound some of the metaphase oscillation measurements by blocking degradation of a regulator of microtubule dynamics (such as cyclin A). The authors should confirm that oscillations are the same in normal metaphase without MG132.

2. Line 77: consider citing work from Bucko et al. (2019 eLife, PMID: 31872801), who also argued that Aurora A phosphorylates Hec1 at kinetochores.

3. Line 359: Asai et al. 2019 (JCB) also proposed that signals at the outer-kinetochore propagate back to mediate centromeric Aurora B activity.

4. In addition to phosphorylating Hec1-S55, Aurora A can promote HURP microtubule binding to stabilize microtubules against depolymerization (Koffa et al. 2006; Wong et al 2008). If Hec1 phosphorylation destabilizes attachments, then these effects of Aurora A activity seem contradictory. Can the authors comment on this point?

5. Line 641: "DeLuca".
I enjoyed reading this manuscript by Iemura et al. on the role of Aurora A-mediated phospho-regulation of Hec1 and its impacts on error correction and chromosome oscillation. Several issues are outlined below that I feel should be addressed prior to publication in JCB.

1) I have a technical concern about assessing chromosome oscillations and other phenomena in MG132-treated cells since this treatment prevents degradation of Cyclin A and affects kMT attachment stability (Kabeche and Compton, Nature, 2013). While the duration of the experiments will be shorter it would be worthwhile to examine if the oscillation properties of the different cell lines in metaphase are the same for MG132-treated and untreated cells.

2) There is also a "chicken and egg" scenario here with regards to chromosome oscillation versus phospho-regulation that I think deserves further fleshing out in the text through additional discussion. The authors data support the idea that kinetochores that venture closer to the pole during chromosome oscillations become more phosphorylated by Aurora A, yet the authors also argue that this phosphorylation promotes the oscillatory activity. I think it would be helpful to further explain how you think the interplay between these two phenomena actually play out at the molecular level.

3) I also have a few concerns about the emphasis on spindle-associated, which the authors are arguing is responsible for their observations, versus the contribution of polar Aurora A kinase activity. Chmatal et al. JCB 2015 found that Mad1 levels (as a proxy for kMT attachment) rose as KTs approached 2-3 microns from the spindle poles. Similarly, Ye et al. JCB 2015 measured an Aurora A activity gradient emanating ~1-2 microns from spindle poles. A fair number of pS55 positive KTs in the images appear to be within several microns of the spindle poles based on the scale bars and the images presented. If this is the case then what is the difference between the spindle-associated (as emphasized here) Aurora A versus a gradient of Aurora A activity that is highest at the spindle poles. In other words, are you simply observing the same phenomenon as Ye at al. and Chmatal et al. but in metaphase cells with more robust chromosome oscillations.

4) Similar to point 3, I do not understand the basis of the statement on lines 363-366 that Aurora A on the spindle, rather than at spindle poles, was responsible for the Hec1-S55 phosphorylation. Of course, spindle poles (as distinct from the centrosome) are part of the spindle and the Aurora A SR mutant looked to be significantly reduced from the entire spindle - including the spindle poles. I think it is more reasonable (see more below for a caveat) to conclude that centrosomal Aurora A, which is still evident in the SR mutant, is not sufficient to phosphorylate S55.

5) However, one major caveat to reaching too strong a conclusion from the SR mutant is that the lack of TPX2 activation likely leads to global reduction in overall Aurora A kinase activity in the mitotic cells. It is worth tempering these conclusions somewhat by taking this into consideration. This could also be addressed by looking at and quantifying active Aurora A kinase localization and levels in the WT versus SR versus KD cells in Figure 3E? Cell Signaling technologies sells a nice antibody that works for immunofluorescence.

6) It should be noted that the results described in lines 317-320 mirror those reported in PtK1 cells by Ye et al. JCB 2015. Increased lagging chromosome, increased merotelic attachments, Aurora A is involved in error correction.

7) I found the data presented in Figure S4 D-F to be quite interesting. Consider moving into a main figure.

8) There were various English language/grammatical issues throughout the text that could be corrected with further editing.
Iemura et al. investigate the potential causes of chromosomal instability (CIN) and aneuploidy in cancer cells. Via timelapse microscopy, they find that cancer cell lines have attenuated chromosome oscillations as compared to non-transformed cells. Via immunofluorescence microscopy, they also find that cancer cell lines have lower levels of serine 55-phosphorylated Hec1 (also called Ndc80), a critical component of the kinetochore’s microtubule-binding interface. They show that serine 55 phosphorylation depends on Aurora A kinase activity and cooperates with serine 69 phosphorylation to enhance metaphase oscillation and suppress chromatid lagging at anaphase.

From a technical perspective, the study is generally well-executed. The development of a chemically degradable form of Aurora A is noteworthy given longstanding issues with the specificity of ATP-competitive Aurora A inhibitors. However I was not convinced of the authors's first major point, and the second point is a modest extension of previously published work (DeLuca et al, JCB (2018); PMID: 29187526). Specific issues are discussed below.

1. In comparing cancer cells against untransformed cells, the authors have to consider the possibility that some differences may not have anything to do with CIN and aneuploidy. One way to detect such CIN-independent changes would be to examine non-CIN cancers. Here, the authors use HCT116 cells, a non-CIN colorectal cancer with a stable near-diploid karyotype, although they do not note this property. Despite lacking CIN, HCT116 cells also exhibit damped metaphase chromosome oscillations (Fig. 1D) and reduced Ndc80 phosphorylation relative to non-cancer controls (Fig. S2G-H). These findings suggest that these phenotypes are cancer- but not CIN-specific, undermining the authors's first major point. Examining a larger panel non-CIN cancer cell lines is needed to know if HCT116 is the exception or the rule.

2. The Ndc80 tail contains multiple consensus sites for Aurora A and Aurora B phosphorylation. Using prometaphase HeLa cells, DeLuca et al (2018) showed that serine 69 phosphorylation is more sensitive to Aurora A inhibitors than Aurora B inhibitors, while the reverse is true for serine 44. Equal sensitivity was noted for serine 55. However all three sites became hypophosphorylated (~90% loss of signal) when cells were co-treated with Aurora A (MLN) and Aurora B (AZD) inhibitors. Here, Iemura et al. focus on metaphase phosphorylation of serine 55, and find that this modification is nearly undetectable in HeLa cells, and present but only partially sensitive to combined Aurora A and Aurora B inhibition in RPE-1 cells (Fig. 2C-D). It is important to note that MLN inhibitor has only slight (~3 fold) selectivity for Aurora A versus Aurora B in HeLa cells, and essentially no selectivity in RPE-1 cells (de Groot et al, Front Oncol (2015); PMID: 26732741). To their credit, the authors engineer an RPE-1 cell line in which endogenous Aurora A can be degraded in an auxin-dependent manner, thereby circumventing this selectivity issue (Fig. 3). However Aurora A degradation reduces metaphase phosphorylation on serine 55 only slightly on its own (20% loss) or in combination with Aurora B inhibition (30% loss; Fig. 3C-D). The magnitude of this reduction is much smaller than the difference between untreated HeLa and RPE-1 cells (80%; Fig. 2C-D). Stated differently, these data suggest that the metaphase pool of serine 55 phosphorylation is either non-dynamic or not generated by either Aurora A or Aurora B. Alternatively, the pS55 antibody may be cross-reacting
with another epitope present in RPE-1 cells. In either case, effect size is small and of uncertain physiologic relevance.

3. In RNAi rescue experiments, the authors find that the S55A Ndc80 mutant does not increase the rate of lagging anaphase chromatids on its own, but instead enhances the rate of lagging when combined with the previously reported S69A mutation (Fig. 6D). This enhancement is an interesting but modest advance over our previous understanding of Ndc80 phosphoregulation, and is somewhat difficult to interpret since prometaphase regulation would also be affected by these mutations.

Minor points:
1. Controls for pS55 antibody specificity (RNAi depletion/rescue, peptide competition/titration) are not provided. As noted above, it is unclear if weak signals detected by the antibody are specific.
2. Graphs are densely labeled and hard to interpret in some cases due to the number of hypotheses being tested. The biological rationale for some comparisons was unclear.
Response to the reviewers’ comments

Changes in the text of the manuscript were highlighted.

Response to Reviewer #1

Major comments:
1. The overall model for how oscillations eliminate incorrect attachments is unclear. First, how does Hec1 phosphorylation promote error correction? Is it by increased microtubule turnover at kinetochores? If so, this should be stated explicitly? Second, is there any selectivity for merotelic attachments? Is the oscillation amplitude larger for merotelics, which would bring them closer to spindle poles? Alternatively, if oscillations affect all kinetochores equally, then the authors should explain how this facilitates error correction.

As suggested by the reviewer, we assume that Hec1 phosphorylation promotes error correction by increasing microtubule turnover at kinetochores, which we stated in discussion (lines 430-432). Our data suggest that Hec1 phosphorylation preferentially occurs on kinetochores at the edge of the metaphase plate, probably due to higher Aurora A localization on K-fibers closer to spindle poles (stated in lines 422-430). Such kinetochores are supposed to be leading kinetochores, where merotelyically-attached microtubules, if any, are polymerizing while correctly-attached microtubules are depolymerizing. It was reported that Hec1 phosphorylation specifically affects turnover of polymerizing microtubules (Long et al. *Curr Biol* 27:1692, 2017), suggesting that merotelyically-attached microtubules on leading kinetochores are selectively destabilized. Even if both merotelyically- and correctly-attached microtubules are destabilized, fewer number of merotelyically-attached microtubules against correctly-attached microtubules, and higher density of microtubules from spindle poles at the correct side for leading kinetochores may facilitate error correction. We mentioned these possibilities in discussion (lines 432-442).

2. The interpretation of the Aurora A S155R experiment is unclear because TPX2 is an Aurora A activator, and thus the mutation is expected to affect kinase activity as well as microtubule binding. Microtubule binding and kinase activity could potentially be decoupled using TPX2 mutants characterized by Zhang et al.
In response to the reviewer’s comment, we examined the involvement of Aurora A on the spindle in metaphase Hec1 phosphorylation using two TPX2 mutants that can bind and activate Aurora A but cannot bind to microtubules, TPX2-F307E F334E H335E (3E) (Zhang et al. *eLife* 6:e30959, 2017) and TPX2-N(aa1-43) (Bayliss et al. *Mol Cell* 12:851, 2003). We established RPE-1 cell lines containing mAID-mClover-TPX2 stably expressing these TPX2 mutants, and observed Hec1 phosphorylation during metaphase after depleting mAID-mClover-TPX2 by adding IAA (Fig. S4, B and C). As shown in Fig. 3 H, spindle localization of Aurora A was restored in mCherry-TPX2-WT-expressing cells, but not in mCherry-TPX2-3E and mCherry-TPX2-N-expressing cells, as expected. In mCherry-TPX2-3E and mCherry-TPX2-N-expressing cells, Hec1-S55 phosphorylation was not restored in contrast to mCherry-TPX2-WT-expressing cells (Fig. 3, I and J), confirming that spindle-associated Aurora A plays a major role in metaphase Aurora A phosphorylation. These results were described in lines 222-250.

It is of note that Aurora A-S155R is actually activated on spindle poles, which was shown by the presence of phosphorylated Aurora A at T288 (Aurora A-pT288) in Aurora A-S155R-expressing cells at comparable level to that in Aurora A-WT-expressing cells (Fig. S4, F and G). In contrast, Aurora A-pT288 was markedly reduced in Aurora A-K162R (kinase-dead)-expressing cells. This is consistent with previous reports that Aurora A can be activated by autophosphorylation, which is different from activation by binding to TPX2 (Zorba et al. *eLife* 3:e02667, 2014, Dodson et al. *J Biol Chem* 287:1150, 2012). Therefore, we think Aurora A-S155R can work as a separation-of-function mutant, which is specifically defective in the activation through TPX2 binding, but not through autophosphorylation (stated in lines 259-265). Combined with the results of the experiments using TPX2 mutants performed in response to the reviewer’s comment, the involvement of spindle-associated Aurora A in the metaphase Hec1 phosphorylation is strongly suggested.

3. For the Aurora A inhibition or depletion experiments in Fig. 6C, it is important to show that the spindle is not disrupted. Otherwise lagging chromosomes might be due to more general spindle problems rather than the authors’ interpretation.
We confirmed that the spindle was maintained when Aurora A and/or B were inhibited in metaphase-arrested cells at the concentration and treatment duration used in the experiment (see Fig. R3 D, MLN 3 h, for the effect of longer treatment), and further observed that spindle microtubules remained after 10 min-cold treatment was comparable in the presence or absence of Aurora A/B inhibitors. We added these results as Fig. S6, D and E, and described in lines 371-375.

Minor points:
1. Using MG132 might confound some of the metaphase oscillation measurements by blocking degradation of a regulator of microtubule dynamics (such as cyclin A). The authors should confirm that oscillations are the same in normal metaphase without MG132.

We observed chromosome oscillation during normal mitotic progression, and compared with the chromosome oscillation in the presence of MG132. Both in RPE-1 and HeLa cells, we found the oscillation amplitude in the absence or presence of MG132 is not significantly different, although the oscillation amplitude tended to be higher in early phase of normal metaphase compared to late metaphase and metaphase in the presence of MG132. These results were shown in Fig. S1, A and B, and Video 2, and described in lines 98-99. We also confirmed that phosphorylation of Hec1-S55 during metaphase is comparable in the presence or absence of MG132 (Fig. R1, A and B). We further compared the level of cyclin A in metaphase RPE-1 and HeLa cells. As shown in Fig. R1, C and D, cyclin A intensity was comparable in the presence or absence of MG132, which was lower than that in prometaphase. Even when we expressed a non-degradable cyclin A mutant (Kabeche et al. Nature 502:110, 2013), amplitude of chromosome oscillation did not significantly change (Fig. R1, E-G), suggesting that cyclin A has a little impact on chromosome oscillation.

2. Line 77: consider citing work from Bucko et al. (2019 eLife, PMID: 31872801), who also argued that Aurora A phosphorylates Hec1 at kinetochores.

According to the reviewer’s suggestion, we cited the paper in the introduction (line 77).

3. Line 359: Asai et al. 2019 (JCB) also proposed that signals at the outer-kinetochore propagate back to mediate centromeric Aurora B activity.
According to the reviewer’s comment, we cited the paper in discussion (line 410), as a mechanism to reduce Aurora B activity on kinetochores upon bi-orientation establishment by increasing phosphatase activity.

4. In addition to phosphorylating Hec1-S55, Aurora A can promote HURP microtubule binding to stabilize microtubules against depolymerization (Koffa et al. 2006; Wong et al 2008). If Hec1 phosphorylation destabilizes attachments, then these effects of Aurora A activity seem contradictory. Can the authors comment on this point?

Phosphorylation by Aurora kinases either facilitates (HURP etc.) or suppresses (Hec1, CENP-E, the Ska complex etc.) the formation of stable kinetochore-microtubule attachments depending on the substrates. Regarding HURP, its localization on spindle is higher near spindle equator, in contrast to Aurora A localization, which is higher near spindle poles. Although we could not obtain a phospho-HURP antibody, spindle localization of HURP did not change by Aurora A inhibition (Fig. R2, A and B), consistent with a recent report (Dudka et al. *Curr Biol* 29:3563, 2019). These data imply that it is less likely that HURP is regulated oscillation-dependent phosphorylation by Aurora A, although whether other Aurora substrates are regulated through oscillation-dependent phosphorylation by Aurora A requires further study. We added these comments in discussion (lines 450-457).

5. Line 641: "DeLuca".

We corrected the typo.
Response to Reviewer #2

1) I have a technical concern about assessing chromosome oscillations and other phenomena in MG132-treated cells since this treatment prevents degradation of Cyclin A and affects kMT attachment stability (Kabeche and Compton, Nature, 2013). While the duration of the experiments will be shorter it would be worthwhile to examine if the oscillation properties of the different cell lines in metaphase are the same for MG132-treated and untreated cells.

We observed chromosome oscillation during normal mitotic progression in RPE-1 and HeLa cells, and compared with the chromosome oscillation in the presence of MG132. We found that the oscillation amplitude in the absence or presence of MG132 is not significantly different, although the oscillation amplitude tended to be higher in early phase of normal metaphase compared to late metaphase and metaphase in the presence of MG132. These results were shown in Fig. S1, A and B, and Video 2, and described in lines 98-99. We also confirmed that phosphorylation of Hec1-S55 during metaphase is comparable in the presence or absence of MG132 (Fig. R1, A and B). We further compared the level of cyclin A in metaphase RPE-1 and HeLa cells. As shown in Fig. R1, C and D, cyclin A intensity was comparable in the presence or absence of MG132, which was lower than that in prometaphase. Even when we expressed a non-degradable cyclin A mutant, amplitude of chromosome oscillation did not significantly change (Fig. R1, E-G), suggesting that cyclin A has a little impact on chromosome oscillation.

2) There is also a "chicken and egg" scenario here with regards to chromosome oscillation versus phospho-regulation that I think deserves further fleshing out in the text through additional discussion. The authors data support the idea that kinetochores that venture closer to the pole during chromosome oscillations become more phosphorylated by Aurora A, yet the authors also argue that this phosphorylation promotes the oscillatory activity. I think it would be helpful to further explain how you think the interplay between these two phenomena actually play out at the molecular level.

Considering that phosphorylation of Hec1-S69 alone can support a certain level of chromosome oscillation in RPE-1 cells, and Hec1-S69 is phosphorylated during metaphase in every cell lines tested irrespective of oscillation amplitude, oscillation amplitude itself may be determined independently of Aurora A activity. Rather, Hec1
phosphorylation at S55 and S69 may allow the intrinsic level of chromosome oscillation by increasing turnover of K-fibers. Therefore, we assume that robust chromosome oscillation is the primary event in non-transformed cell lines, and resulting Hec1-S55 phosphorylation may reinforce chromosome oscillation. We added these argument in discussion (lines 466-473), and changed the description in abstract (lines 31-32).

3) I also have a few concerns about the emphasis on spindle-associated, which the authors are arguing is responsible for their observations, versus the contribution of polar Aurora A kinase activity. Chmatal et al. JCB 2015 found that Mad1 levels (as a proxy for kMT attachment) rose as KTs approached 2-3 microns from the spindle poles. Similarly, Ye et al. JCB 2015 measured an Aurora A activity gradient emanating ~1-2 microns from spindle poles. A fair number of pS55 positive KTs in the images appear to be within several microns of the spindle poles based on the scale bars and the images presented. If this is the case then what is the difference between the spindle-associated (as emphasized here) Aurora A versus a gradient of Aurora A activity that is highest at the spindle poles. In other words, are you simply observing the same phenomenon as Ye at al. and Chmatal et al. but in metaphase cells with more robust chromosome oscillations.

We agree that what we are observing in metaphase is basically the same phenomenon as reported by Ye et al. and Chmatal et al. in prometaphase, which depends on Aurora A activity gradient. However, we assume that this “gradient” is not just a density gradient of soluble pool of Aurora A turning over on spindle poles, but rather a gradient of spindle-associated Aurora A formed by dynein-dependent transport of TPX2 (Ma et al. Mol Biol Cell 21:979, 2010) due to the following reasons; i) Aurora A localization gradient is only seen on the spindle, but not all the direction around spindle poles (Fig. 3 F), ii) Aurora A localization on the spindle becomes apparent in metaphase when mature K-fibers are formed (Fig. S4 H). Furthermore, we confirmed that Aurora A-S155R, which cannot localize to the spindle microtubules, is active on spindle poles, judged by phosphorylation of Aurora A-T288 (Fig. S4, F and G, lines 259-265), in agreement with previous reports that Aurora A can be activated by autophosphorylation, which is different from activation by binding to TPX2 (Zorba et al. eLife 3:e02667, 2014, Dodson et al. J Biol Chem 287:1150, 2012). Therefore, we favor the idea that Aurora A on the spindle is mainly responsible for Hec1 phosphorylation. We included the above discussion in the revised manuscript (lines 422-430). However, we attenuated
our conclusion considering the possibility that Aurora A on spindle poles can also plays a role in Hec1 phosphorylation, through either diffusion or turning over to the spindle-pool of Aurora A (lines 28, 84, 169, 265, 391, 414, and 1074).

4) Similar to point 3, I do not understand the basis of the statement on lines 363-366 that Aurora A on the spindle, rather than at spindle poles, was responsible for the Hec1-S55 phosphorylation. Of course, spindle poles (as distinct from the centrosome) are part of the spindle and the Aurora A SR mutant looked to be significantly reduced from the entire spindle - including the spindle poles. I think it is more reasonable (see more below for a caveat) to conclude that centrosomal Aurora A, which is still evident in the SR mutant, is not sufficient to phosphorylate S55.

We think Aurora A on the spindle, which is activated and recruited to microtubules through binding to TPX2, is a major Aurora A population to encounter and phosphorylate Hec1 at kinetochores. However, we agree that it is possible that Aurora A at spindle poles, residing at the peak of the gradient, can phosphorylate Hec1, either through diffusion or through turning over to the pool on the spindle. Therefore, we attenuated our conclusion (“Aurora A residing on the spindle is mainly responsible for the Hec1-S55 phosphorylation”), according to the reviewer’s comment in lines 28, 84, 169, 265, 391, 414, and 1074.

5) However, one major caveat to reaching too strong a conclusion from the SR mutant is that the lack of TPX2 activation likely leads to global reduction in overall Aurora A kinase activity in the mitotic cells. It is worth tempering these conclusions somewhat by taking this into consideration. This could also be addressed by looking at and quantifying active Aurora A kinase localization and levels in the WT versus SR versus KD cells in Figure 3E? Cell Signaling technologies sells a nice antibody that works for immunofluorescence.

According to the reviewer’s comment, we observed the localization of Aurora A phosphorylated at the activation-loop (Aurora A-pT288). We found that phosphorylation of T288 in Aurora A-S155R at spindle poles is comparable to that in Aurora A-WT, while that in Aurora A-K162R is reduced (Fig. S4, F and G). The result is in agreement with previous reports that Aurora A can be activated by autophosphorylation, which is different from activation by binding to TPX2 (Zorba et al.
We described the result in lines 259-265. Therefore, we think Aurora A-S155R can work as a separation-of-function mutant, which is specifically defective in the activation through TPX2 binding, but not through autophosphorylation. Furthermore, we examined TPX2 mutants (TPX2-3E and TPX2-N) that binds to Aurora A, but not to microtubules, and found that these mutants cannot restore the Hec1-S55 phosphorylation to the wild type level (Fig. 3, H-J, Fig. S4, B and C). Although these data strongly suggest the involvement of spindle-associating TPX2 in Hec1-S55 phosphorylation, we tempered our conclusion according to the reviewer’s comments, considering that we cannot fully exclude the possibility that centrosomal Aurora A has something to do with the Hec1 phosphorylation during metaphase, either directly or indirectly (lines 28, 84, 169, 265, 391, 414, and 1074).

6) It should be noted that the results described in lines 317-320 mirror those reported in PtK1 cells by Ye et al. JCB 2015. Increased lagging chromosome, increased merotelic attachments, Aurora A is involved in error correction.

We agree with the reviewer’s comment, and stressed in the revised manuscript that the Aurora A-dependent error correction in metaphase mirrors the similar phenomenon in prometaphase cells reported by Ye et al (lines 420-422).

7) I found the data presented in Figure S4 D-F to be quite interesting. Consider moving into a main figure.

In response to the reviewer’s comment, we moved the data to a main figures, Fig. 3, E-G.

8) There were various English language/grammatical issues throughout the text that could be corrected with further editing.

According to the reviewer’s comment, we carefully edited the entire manuscript.
Response to Reviewer #3

Major points:
1. In comparing cancer cells against untransformed cells, the authors have to consider the possibility that some differences may not have anything to do with CIN and aneuploidy. One way to detect such CIN-independent changes would be to examine non-CIN cancers. Here, the authors use HCT116 cells, a non-CIN colorectal cancer with a stable near-diploid karyotype, although they do not note this property. Despite lacking CIN, HCT116 cells also exhibit damped metaphase chromosome oscillations (Fig. 1D) and reduced Ndc80 phosphorylation relative to non-cancer controls (Fig. S2G-H). These findings suggest that these phenotypes are cancer- but not CIN-specific, undermining the authors's first major point. Examining a larger panel non-CIN cancer cell lines is needed to know if HCT116 is the exception or the rule.

According to the reviewer’s comment, we addressed the amplitude of chromosome oscillation and the level of Hec1-S55 phosphorylation in non-CIN cancer cell lines. First of all, we have to point out that HCT116 cells maintained in our lab show an elevated level of CIN compared to previous reports, as shown in Fig. S6, A and B in the original manuscript, suggesting the change of cellular property during culture. Therefore, we obtained HCT116 cells from ATCC, and also examined two other non-CIN cancer cell lines, HCT-15 and DLD-1. We found that HCT116 cells obtained from ATCC as well as other two non-CIN cell lines show reduced levels of CIN, judged by the micronucleation rates and karyotypic diversity, compared to our previous HCT116 cells and other CIN cancer cell lines. However, the level of CIN was still higher than non-transformed cell lines, indicating that even “non-CIN” cancer cell lines exhibit a certain level of CIN. Intriguingly, the amplitude of chromosome oscillation and the level of Hec1-S55 phosphorylation in these non-CIN cell lines were also higher than those in CIN cancer cell lines, but lower than those in non-transformed cell lines. These results support our model that the amplitude of chromosome oscillation and the level of Hec1-S55 phosphorylation correlates with the level of CIN. We included these results in the revised manuscript (Fig. 1, C and D, Fig. 2, E and F, Fig. S1, C-E, G-J, lines 106-110, 156-158, 503-508).

2. The Ndc80 tail contains multiple consensus sites for Aurora A and Aurora B phosphorylation. Using prometaphase HeLa cells, DeLuca et al (2018) showed
that serine 69 phosphorylation is more sensitive to Aurora A inhibitors than Aurora B inhibitors, while the reverse is true for serine 44. Equal sensitivity was noted for serine 55. However all three sites became hypophosphorylated (~90% loss of signal) when cells were co-treated with Aurora A (MLN) and Aurora B (AZD) inhibitors. Here, Iemura et al. focus on metaphase phosphorylation of serine 55, and find that this modification is nearly undetectable in HeLa cells, and present but only partially sensitive to combined Aurora A and Aurora B inhibition in RPE-1 cells (Fig. 2C-D). It is important to note that MLN inhibitor has only slight (~3 fold) selectivity for Aurora A versus Aurora B in HeLa cells, and essentially no selectivity in RPE-1 cells (de Groot et al, Front Oncol (2015); PMID: 26732741). To their credit, the authors engineer an RPE-1 cell line in which endogenous Aurora A can be degraded in an auxin-dependent manner, thereby circumventing this selectivity issue (Fig. 3). However Aurora A degradation reduces metaphase phosphorylation on serine 55 only slightly on its own (20% loss) or in combination with Aurora B inhibition (30% loss; Fig. 3C-D). The magnitude of this reduction is much smaller than the difference between untreated HeLa and RPE-1 cells (80%; Fig. 2C-D). Stated differently, these data suggest that the metaphase pool of serine 55 phosphorylation is either non-dynamic or not generated by either Aurora A or Aurora B. Alternatively, the pS55 antibody may be cross-reacting with another epitope present in RPE-1 cells. In either case, effect size is small and of uncertain physiologic relevance.

In response to the reviewer’s comments, we carefully re-evaluated the signal level of the Hec1-S55 phosphorylation in metaphase.

First, we confirmed the specificity of the phospho-Hec1-S55 signal by observing the disappearance of the signal in the presence of a blocking peptide (Fig. S2, A and B), and by Hec1 RNAi, which was recovered by expressing wild type Hec1, but not by a Hec1 mutant in which serine 55 was mutated to alanine (Hec1-S55A) (Fig. S2, C and D). These results were described in lines 131-135.

Next, we identified a couple of reasons that could explain the partial reduction of the phospho-Hec1-S55 signal when Aurora A was inhibited or depleted.

1) Effect of background signal
In the original manuscript, we quantified the phospho-Hec1-S55 signal overlapping with the Hec1 signal, and presented the relative value against Hec1 signal without subtracting background. When we subtracted the background signal, which was represented by the signal of the same area at the cytoplasm, the reduction of the
phospho-Hec1-S55 signal by Aurora A inhibition became much more obvious (~70% loss of signal; Fig. R3 A). We realized that we had been overlooking the effect of background signal level, especially when evaluating the weak signal, and amended all the quantification data (phospho-Hec1-S55, S69, and total Hec1) by subtracting the background signal in the revised manuscript and described the quantification procedure in lines 626-629.

2) Difference in the fixation method
Compared to the effect of Aurora A inhibition to the reduction of phospho-Hec1-S55 signal (e.g. Fig. 2 D), the effect of Aurora A depletion by auxin-dependent degradation was modest (~50% loss of signal) even when the background signal was subtracted (e.g. Fig. 3D). This can be explained by the difference in the cell fixation method used in these experiments. When the effect of Aurora A inhibition was examined, cells were fixed with methanol that gave rise to a good signal-to-noise ratio for the phosphorylation signal (Fig. 2, C and D, Fig. R3 A). On the other hand, we had to fix the cells with paraformaldehyde when Aurora A was depleted, because Aurora A-mAID-mClover signal was not detectable when cells were fixed with methanol. In cells fixed with paraformaldehyde, the signal-to-noise ratio was lower, which would be a reason why the effect of Aurora A depletion was less clear (Fig. 3, C and D, Fig. R3 C).

3) Degree of Aurora A inhibition
Even when the background signal was subtracted in RPE-1 cells treated with an Aurora A inhibitor, the level of the phospho-Hec1-S55 level was still higher compared to the level in HeLa cells (Fig. 2 D). Regarding the point, we surmised that it is due to the residual Aurora A activity, in addition to intrinsic difference of the background level between cell lines. Supporting the possibility, the phospho-Hec1-S55 level in RPE-1 cells was comparable to the level in HeLa cells when cells were treated with the Aurora A inhibitor for 3 h instead of 1 h treatment used in our study (Fig. R3, D and E). However, as longer Aurora A inhibition affected the spindle integrity (Fig. R3 D, MLN 3 h), we could not inhibit Aurora A activity further in our experiments.

3. In RNAi rescue experiments, the authors find that the S55A Ndc80 mutant does not increase the rate of lagging anaphase chromatids on its own, but instead enhances the rate of lagging when combined with the previously reported S69A mutation (Fig. 6D). This enhancement is an interesting but modest advance over our previous understanding of Ndc80 phosphoregulation,
and is somewhat difficult to interpret since prometaphase regulation would also be affected by these mutations.

As the reviewer pointed out, the effect of Hec1 mutations on mitotic fidelity is not confined to metaphase in the experiment shown in Fig. 6 D, because we could not replace endogenous Hec1 with the Hec1 mutants specifically in metaphase. We clarified the point in lines 382-383, and changed the description in abstract (line 34) to avoid confusion. Metaphase-specific effect of Aurora A on mitotic fidelity, however, is shown in Fig. 6 C, where Aurora inhibitors was added after metaphase arrest. Therefore, compared to previous findings, our findings are unique in terms of metaphase-specific effect of Aurora A on mitotic fidelity, which is defective in cancer cell lines depending on the reduced Hec1-S55 phosphorylation.

Minor points:
1. Controls for pS55 antibody specificity (RNAi depletion/rescue, peptide competition/titration) are not provided. As noted above, it is unclear if weak signals detected by the antibody are specific.

We confirmed the specificity of the phospho-Hec1-S55 signal in metaphase by observing the disappearance of the signal in the presence of a blocking peptide, as well as the disappearance or the signal by Hec1 RNAi, which was recovered by expressing wild type Hec1, but not by expressing a Hec1 mutant in which serine 55 was mutated to alanine (Hec1-S55A) (Fig. S2, A-D). These results were described in lines 131-135. We further observed the reduction of the phosphorylation signal when cells were treated with the Aurora A inhibitor for a longer period (Fig. R3, D and E).

2. Graphs are densely labeled and hard to interpret in some cases due to the number of hypotheses being tested. The biological rationale for some comparisons was unclear.

According to the reviewer’s comment, we amended the graphs in Fig. 3 D, Fig. S1 C, S1 D, S1 H, S1 I, S1 J, S5 E, S6 F to improve clarity by showing statistical significance between samples with biological rationale.
April 12, 2021

RE: JCB Manuscript #202006116R

Prof. Kozo Tanaka
Tohoku University
4-1 Seiryo-machi, Aoba-ku
Sendai 980-8575
Japan

Dear Prof. Tanaka,

Thank you for submitting your revised manuscript entitled "Chromosome oscillation promotes Aurora A-dependent Hec1 phosphorylation and mitotic fidelity". The reviewers are now satisfied with the revision and recommend minor text edits for clarity and to improve the scholarly discussion of other work. We would be happy to publish your paper in JCB pending final revisions necessary to address the remaining reviewer comments and to meet our formatting guidelines (see details below).

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

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   - Please include a summary statement on the title page of the resubmission. It should start with "First author name(s) et al..." to match our preferred style.

2) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Please add scale bars to 2ACE (magnifications), 3I (mags), 4CGK (mags), 6A (mags), S2ACEGIK (mags), S3EH (mags), S5DH (mags)

3) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends.

4) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions in the text for readers who may not have access to referenced manuscripts.
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   - Please include species and source for all antibodies, including secondary, as well as catalog numbers/vendor identifiers if available.
   - Sequences should be provided for all oligos: primers, si/shRNA, gRNAs, etc.
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  c. Temperature
  d. Imaging medium
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  f. Camera make and model
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Thank you for this interesting contribution, we look forward to publishing your paper in the Journal of Cell Biology.

Sincerely,

Hironori Funabiki, PhD
Monitoring Editor, Journal of Cell Biology

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

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

The authors have addressed my concerns by (1) presenting a model for error correction based on their findings, (2) performing additional experiments using TPX2 mutants that fail to bind MTs, and (3) showing that the spindle is not disrupted in their Aurora A inhibition experiments. I recommend publication.

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

The authors have addressed my comments in this resubmitted manuscript. One of my major concerns related to interpretation of the role of TPX2. The new observation that there are normal levels of active Aurora A at centrosomes in the absence of TPX2 binding is interesting and supports the idea that the kinase can become locally activated independent of TPX2 potentially through autophosphorylation. Yet this population of Aurora A does not appear to contribute to polar phosphorylation of S55. The fact that polar kinetochores were not efficiently phosphorylated at HEC1-S55 in both the Aurora A-SR mutant and either the TPX2-3E or "N" mutants supports the authors conclusion that spindle-associated/TPX2-activated Aurora A is the major contributor to the observed phenomenon.

Mangal et al. (JCB, 2018) should be cited since a similar mechanism (MT-bound TPX2-based activation of Aurora A kinase near spindle poles) plays a role in removing contractility components from the polar cortex in C. elegans.

Reviewer #3 (Comments to the Authors (Required)):

The authors have responded to previous critiques with additional experiments and explanations for discrepancies. These changes largely address my concerns with the previous version of the study.