JCB

Delineating the contribution of Spc105-bound PP1 to spindle checkpoint silencing and kinetochore microtubule attachment regulation

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Re: JCB manuscript #201810172

Dr. Ajit Joglekar
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Dear Dr. Joglekar,

Thank you for submitting your manuscript entitled "Minimization of cross-talk between Spindle Assembly Checkpoint silencing and error correction" to Journal of Cell Biology. The manuscript has now been assessed by expert reviewers, whose reports are appended below. Unfortunately, after an assessment of the reviewer feedback, our editorial decision is against publication in JCB.

As you will see, the reviewers do not find that your conclusions are sufficiently supported by your data for publication in JCB, therefore I do not have the level of reviewer support that I would need to proceed further with the paper. I do realize that significant further work and expansion might convincingly address some of these issues, but I am hesitant to encourage you to work towards the aim of further consideration at JCB. The level of reviewer criticism makes it impossible for me to guarantee that we will be able to invite resubmission, even after revision. Therefore, it does seem that it will be best for you to consider another journal for this work.

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I am sorry the decision for JCB is not more positive, but hope that you find the reviews constructive. Of course, this decision does not imply any lack of interest in your work and we look forward to future submissions from your lab.

Thank you for your interest in Journal of Cell Biology.

Sincerely,

Arshad Desai, PhD
Monitoring Editor

Andrea L. Marat, PhD
Scientific Editor

Journal of Cell Biology

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

In their manuscript titled "Minimization of cross-talk between Spindle Assembly Checkpoint silencing and error correction", authors Roy, Joglekar and colleagues investigate the functions of the N-terminal tail of Spc105/Knl1 in chromosome segregation using budding yeast as a model system. They examine two previously uncharacterized regions of the budding yeast Spc105 that are rich in basic residues. Mutants in these basic patches have phenotypes reminiscent of a milder version of mutants in this region that disrupt phosphatase binding, resulting in a metaphase delay due to decreased silencing of the spindle assembly checkpoint. The authors then go on to show that these mutants are able to suppress the phenotype of mutants in the chromosome biorientation pathway. Furthermore, artificially targeting of the phosphatase to Spc105 during the error correction process decreases the rate of chromosome biorientation.

Together, these results suggest two key advances. Firstly, that PP1 recruitment to Spc105 affects chromosome biorientation in a manner independently of the two known pathways: checkpoint silencing and Sgo1 recruitment to the inner centromere. Secondly, that this activity must be regulated to prevent premature stabilization of incorrect attachments.

However, it is not clear to me that any of the phenotypes shown are independent of this known checkpoint-silencing function of PP1-Spc105. It has been previously demonstrated that increased time in metaphase will increase the accuracy of chromosome segregation by giving the chromosomes more time to make proper attachments (nicely demonstrated in Munoz-Barrera 2015 for example). Unless the authors can convincingly demonstrate that the phenotypes they observe are independent of the known functions of Spc105-recruited PP1, I recommend that this paper be published in journal with less emphasis on novelty.

Major concerns:

1. To elaborate on the main point above, the phenotypes observed for the basic patch mutants include increased localization of the checkpoint proteins Bub3 and Mad1 (Fig. 1E), a cell-cycle delay (Fig. 2C), and rescue of mutants in the biorientation pathway (Fig. 3B and 4E). All of these phenotypes can be explained by a delay in metaphase due to slower SAC silencing.

The experiment that strongly argues against this interpretation is the rescue of sgo1D benomyl sensitivity with a RASA mad2D triple mutant. Unfortunately for the two strains shown, one of them showed rescue and the other one did not, making interpretation of this result rather murky. A clear

demonstration of sgo1D rescue on benomyl plates independent of the checkpoint for both the RASA and BPM mutants would go a long way to support the claims of a more direct role in chromosome biorientation.

I would recommend combining the BP and RASA mutants with the spc105-6A mutant that is used as a control in the benomyl-sensitivity experiments. This should demonstrate that the rescue is independent of both the checkpoint silencing and Sgo1-recruitment pathways.

The fact that the RASA and BPM mutants very clearly show rescue of mad2D on benomyl plates does indicate some role in chromosome segregation outside of the checkpoint function, which is quite interesting. However, this additional role is not necessarily in error correction.

I believe that the checkpoint-silencing role could also explain the biorientation phenotype in Figure 5A. Too much phosphatase activity could prevent checkpoint activation and allow premature progression into anaphase with misattachments. I could not find any mention that this experiment was conducted in a metaphase arrest condition such as cdc20 depletion, which would help alleviate this concern. Premature progression into anaphase could also explain the increase in spindle length.

2. I don't see how the "kinetochore biorientation" assay used in figures 1D and 4B is measuring biorientation. This assay is looking as all of the kinetochores at once, such that chromosomes that have not yet bioriented would not be readily discernable from those that have bioriented. Measuring the timing of biorientation would require observing individual kinetochores, or in extreme cases, looking as kinetochore asymmetry as is shown in figure 5A.

On a similar note, the authors claim that the Bub3 and Mad1 localization are on "bioriented kinetochores" and that the cells are "in metaphase". I see no indication of cell cycle synchronization, so how do the authors know that all of the kinetochores on these spindles are already bioriented and that the chromosomes are not still in the process of error correction?

3. The in vitro data strongly suggests that mutations in the basic patch strongly affect microtubule binding directly and have no measurable effect on PP1 binding. I found it very surprising that the authors conclude from this data that "Any potential microtubule-binding activity of the basic patch is unlikely to be important for the phenotypes discussed here." It seems that a model where the microtubule-binding activity of the basic patch(es) somehow acts together with the phosphatase-binding activity of the RVSF motif would be more in line with the presented data.

Minor concerns:

1. The authors state that "However, this inhibition of PP1 recruitment by Aurora B is mainly thought enhance the recruitment of SAC proteins, and contribute to robust SAC signaling (Nijenhuis et al., 2014). The possibility that this inhibition is crucial for efficient error correction has not been considered." To say that something has not been considered is strange, and it didn't take me long to find this in the literature: "However, these data may still be consistent with a role for both PP1-Knl1 and PP2A-B56 in antagonizing Aurora B, since the negative effects of PP1-Knl1 mutation could simply be masked by a compensatory increase in kinetochore PP2A-B56 (as a result of phosphatase cross-talk; see Figure 3H). In agreement with this hypothesis, rescuing PP1-Knl1 following PP2A-B56 depletion is sufficient to reduce Aurora B activity and improve chromosome alignment (Nijenhuis et al., 2014)." from a review by Adrian Saurin this year. This sentence should be

rephrased.

2. Labeling of the Ndc80 complex is typically done through Nuf2 (Joglekar et al 2006, Joglekar et al 2008). Is this because labeling Ndc80 directly leads to a chimeric protein that is not fully functional? It seems possible that Glc7 targeting to Ndc80 does not affect chromosome segregation because the tagged Ndc80 is being outcompeted for kinetochore occupancy by the wild-type.

3. The example images in Fig. 4E are very difficult to interpret. The weak contrast and merging of the two colors make the centromere dots very hard to see.

4. The authors state that "Spc105BPM improves the accuracy of chromosome segregation". This implies that accuracy is somehow improved under normal/all conditions, not that is suppresses the segregation errors caused by a specific mutation (sgo1D).

5. The authors refer to the Spc105D80-128 mutant as "the basic patch and surrounding residues", however this deletion starts directly after the RVSF PP1-binding motif. Based on the fact that the basic patch has no effect on PP1 binding in vitro but the RVSF motif does, it seems more likely that this is a partial disruption of the RVSF-binding function, not an increased disruption of the basic patch. I would describe this mutant as "basic patch plus RVSF-adjacent."

6. The graph in figure 2C measures the time from G1 to anaphase, yet the manuscript states that it measures "metaphase-to-anaphase".

7. The authors state that "Thus, spc105BPM acts as a gain of function mutation by reducing Glc7 recruitment to the kinetochore via Spc105." This statement is not consistent with the in vitro binding data.

8. I am very confused by these two juxtaposed statements: "The stabilization of bipolar attachments is achieved by an Spc105-independent Glc7 activity. Second, our data imply that Glc7 recruited by the RVSF motif interferes with error correction" These two concepts seem contradictory. Is this implying that Glc7-Spc105 interferes with error correction in a mechanism other that stabilizing MT-KT connections? Are the authors trying to say that Glc7-Spc105 can stabilize connections, but ordinarily doesn't?

Overall, the manuscript would greatly benefit from more clarity in the writing.

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

The KNL1 family of kinetochore proteins play a central role in co-ordinating chromosome bi-orientation and spindle checkpoint (SAC) signalling. Members of the KNL1 family contain binding sites for microtubules, type-1-phosphatase (PP1) and components of the SAC machinery (particularly Bub3-Bub1). In this study Roy and colleagues propose that a basic patch motif (BPM), located at the N-terminal region of Spc105 (budding yeast homologue of KNL1), binds both microtubules and aids the recruitment of Glc7. Secondly, they propose that delayed interaction of Glc7 with Spc105 ensures chromosome bi-orientation before SAC silencing. Unfortunately, the experimental logic and presented evidence do not support the conclusions of the manuscript.

Major issues.

A previous study in C.Elegans identified a microtubule binding site in KNl1 immediately N-terminal, but distinct from, the G/SILK PP1-binding motif (Espeut et al., 2012. J. Cell Biol. 196, 469). Inspired by this study the authors show that mutation of four basic residues RRRK (a.a. 101-104) in budding Spc105 (Spc105-BPM) inhibits interaction with microtubules in vitro (Figure 1C). This is convincing. Mutation of the basic patch does not, however, influence interaction with Glc7 (PP1) (Text page 6; Figure S1C). As such it would seem that the microtubule binding and PP1 binding sites in Spc105 can be separated, consistent with evidence from C.Elegans.

However, the authors argue that the basic patch does influence binding to PP1 by showing that deletion of a larger region of Spc105 (D80-120) displays reduced PP1 binding (Figure 1C). Unfortunately, recent structural data reveals that a hydrophobic region immediately C-terminal to the conserved RRVSF motif of human KNL1 interacts with the catalytic subunit of PP1 (Bajaj et al., 2018, Structure 26, 1327). Since the Spc105(D80-120) mutant removes sequences immediately C-terminal to the RRVSF motif, the conclusion that the basic patch contributes to binding of PP1 to Spc105 is not supported. This undermines the interpretation and conclusion of experiments in the rest of the paper.

Indeed, it is hard to rationalise why mutation of a region of Spc105 that is involved in binding PP1 would lead to increased resistance to microtubule destabilising agents and improve the efficiency of chromosome bi-orientation. One would expect outer kinetochore proteins to be more highly phosphorylated (less phosphatase bound) leading to more unstable kinetochore-microtubule interactions. Bajaj and colleagues recently shown that the interaction of PP1 and microtubules with human KNL1 is mutually exclusive (Bajaj et al., 2018, Structure 26, 1327). If this competition occurs in vivo then the Spc105-BPM may infact bind more PP1 leading to stabilisation of microtubule-kinetochore interactions. Regardless, a tangible molecular explanation for the effect of the spc105-BPM mutant on microtubule stability and chromosome bi-orientation is currently missing.

The authors should note that both the S/GILK and RRVSF motifs in human KNL1 and fission yeast Spc7 contribute to association of PP1 in vitro, although the interaction of PP1 with the S/GILK motif is weaker than with the RRVSF motif (Meadows et al., 2011, Developmental Cell 20, 739; Bajaj et al., 2018, Structure 26, 1327). Mutation or deletion of the RRVSF motif in either budding yeast Spc105 or fission yeast Spc7 causes cell cycle arrest due to hyperactivation of the SAC; namely the lethality is rescued by deletion of Mad2 or Mad3, components of MCC. This suggests that interaction of PP1 with the RRVSF silences the SAC (Rosenberg et al., 2011, 21, 942; Meadows et al., 2011, Developmental Cell 20, 739). However, the GILK motif in Spc7 is also essential for viability in fission yeast but this is not bypassed by inactivation of Mad2 or Mad3 (Meadows et al., 2011, Developmental Cell 20, 739). Since in the authors hands Glc7 (PP1) binds weakly to the Spc105(RASA) mutant (Figure S1C) the authors may like to consider whether the GILK motif in Spc105 contributes to PP1 binding and whether this association is more important for chromosome bi-orientation than for SAC signalling. Differential functions, regulation and timing for the association of PP1 to the G/SILK and RRVSF motifs, respectively, may provide a more compelling explanation as to how Spc105-PP1 co-ordinates chromosome bi-orientation and SAC silencing.

Other issues

The authors suggest that phosphorylation of serine and threonine residues to the C-terminus of the basic patch might influence microtubule and/or PP1 binding (Figure 4F). This referee could find no data showing that these residues are phosphorylated in the Craig et al., 2004 paper or in other phospho-proteome databases (e.g. https://thebiogrid.org/33158/protein). Direct evidence should be

presented that these residues are phosphorylated during mitosis or the mutant data should be removed.

It is not terribly surprising that mutation of the basic patch does not further increase Bub3 levels at the kinetochore in the presence of nocodazole (Figure 2A) as there would be no microtubules present and Mps1 would be maximally active.

The title of Figure legend 2 is incorrect. This figure shows no data on error correction.

The recent paper from Page and colleagues (Bajaj et al., (2018) Structure 26, 1327-1336) detailing the structure of the KNL1-PP1 complex should be properly discussed and referenced.

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

In this manuscript (Minimization of cross-talk between Spindle Assembly Checkpoint silencing and error correction), the authors investigate kinetochore functions of KNL1/Spc105-recruited PP1/Glc7 in budding yeast. The large kinetochore scaffold protein Spc105 (KNL1 in human cells) recruits the phosphatase PP1/Glc7 to kinetochores via its N-terminal RVSF motif. In many organismal systems, it has been demonstrated that PP1 is in involved in both stabilizing kinetochore-microtubule attachments and silencing the SAC. Because cells need to silence the SAC only after kinetochore-microtubules are correctly formed and stabilized, the authors here reason that these two functions of PP1 need to be carried out sequentially. To investigate how this might be facilitated the authors test if Spc105-recruited Glc7 has a role in kinetochore-microtubule attachment regulation. The major conclusions they draw are: (1) Spc105 recruits Glc7 for SAC silencing but not kinetochore-MT attachment regulation and therefore, there must be an Spc105-independent pool of Glc7 that functions to regulate kinetochore-MT attachments; and (2) kinetochores must delay Glc7 recruitment until correct attachments are made so that the SAC is not pre-maturely silenced. The issue here is an important one: if there is only mode of recruitment for Glc7, attachment security and SAC silencing would occur simultaneously rather than sequentially. Thus, a second Glc7/PP1 pool is likely required for stabilization of attachments and is recruited to the kinetochore-MT interface based on criteria that are different than those required for recruitment to Spc105 and SAC silencing.

I think the authors have developed an important new model for PP1/Glc7 recruitment and function at kinetochores. However, many of the results are redundant with past findings (Rosenberg et al., 2011; London et al., 2012; Nijenhuis et al., 2014; Espeut et al., 2012; Liu et al., 2010). What is actually new here is the re-framing and evaluation of the results in the context of this new model. With that being said, there is some new information provided, and the study should be of interest to the field. However, there are some concerns that preclude recommendation of the manuscript for publication in the JCB in its current form. For example, the first third of the paper uses a mutant of Spc105 as a tool to reduce PP1/Glc7 levels at kinetochores, however, it is not convincing that this mutant has any role in Glc7 recruitment. In addition, a major premise of the study is that reduction of PP1/Glc7 at kinetochores results in "faster" kinetochore bi-orientation, which is used a base assumption for much of the paper. As presented, the data do not necessarily support this conclusion. These and other issues that should be addressed are described in detail below.

Major comments:

(1) The authors conclude early on that the Spc105 basic patch has an important role in recruiting Glc7 to kinetochores, but the data supporting this conclusion are not convincing. Clearly the basic patch binds MTs - this has been shown in other systems and the authors confirm those findings nicely here. But the evidence demonstrating that this motif recruits Glc7 is not compelling. For example: The authors carry out an experiment (shown in Supplemental figure 1C) to "directly test whether the basic patch contributes to Glc7 recruitment by Spc105." Here, they quantify the amount of Myc-Glc7 that is pulled out of yeast lysates with beads coated with various Spc105 fragments. While they show clearly that Spc105-RASA (a mutant that cannot bind Glc7) is impaired for Glc7 binding, Spc105-BPM is not. This seems to be clear evidence that the basic patch does not function to recruit Glc7 to Spc105.

In this experiment, the authors also use an Spc105 mutant deleted for amino acids 80-128, which removes the basic patch and an additional 45 amino acids. This mutant, unlike the basic patch mutant, is reduced for Glc7 binding. However, from figure 1B, it looks like amino acid 80 is either the "F" of the RVSF motif or the residue immediately following. Thus, it's not clear if the RVSF mutant is functional in this Spc105 fragment.

(2) The authors find that Spc105-BPM expressing cells exhibit enhanced recruitment of Bub3 to kinetochores (Figure 1F). The two hypotheses they present to explain this are: (1) mutating the basic patch results in decreased Glc7, which leads to decreased dephosphorylation (increased/retained phosphorylation) of the MELT motifs thus and high Bub3 recruitment; (2) mutating the basic patch prevents Spc105 from binding MTs, and this prevents a conformational change that would normally occur upon MT binding that results in structural reorganization of the kinetochore, preventing Mps1 from phosphorylating the MELT motifs. To distinguish between the two, they generate constructs in which they add back a basic patch alone with "surrounding residues" (to presumably rescue MT binding) or a basic patch plus the RVSF motif (canonical Glc7 recruitment domain). They find that the basic patch alone does not suppress Bub3 recruitment but the basic patch plus RVSF does. They then conclude that the MT binding activity of the basic patch cannot be responsible for the observed results. Two things here: (1) Did the authors test that adding back the basic patch indeed rescued MT binding? If not, the experiment is inconclusive. (2) The authors conclude that the data "strongly suggest that the basic patch and the RVSF motif act together to recruit Glc7 activity in the yeast kinetochore..." Because the basic patch did not suppress Bub3 retention but RVSF plus the basic patch did, this result indicates that RVSF is important for recruiting Glc7, but doesn't address the role of the basic patch (except that it is not sufficient to recruit Glc7). It would be useful for the authors to add back RVSF (plus surrounding residues) alone and compare the retention of Bub3 to that of adding back RVSF plus the basic patch. If the basic patch has a role in Glc7 recruitment, one would predict a differential response to Bub3 retention.

(3) Throughout the paper, the authors propose and conclude that the basic patch is important for recruiting Glc7 to kinetochores. In the first three figures of the paper, the authors utilize the basic patch mutant to probe how decreased levels of Glc7 affect various aspects of kinetochore function. However, based on the above two points, the authors cannot conclude that the basic patch has a clear role in Glc7 recruitment. Because of this and the fact that the basic patch has an additional function in microtubule binding, the binding patch mutant is not appropriate as a tool to test biological outcomes of reduced Glc7 recruitment.

(4) Another major conclusion the authors draw is that because the binding patch mutant reduces

Glc7 recruitment to kinetochores, this results in "faster kinetochore bi-orientation." It is not clear that this is the case from the data presented in Figure 1D (and for Figure 4B). They show SPB-kinetochore and kinetochore-kinetochore separation at two time-points after release from a G1 arrest. A couple of things here: (1) There is no rate information shown. Is the conclusion that they bi-orient faster drawn from the fact that the two kinetochore masses are farther apart at the two time points? (2) It is also not clear if the kinetochores are properly bi-oriented. The Danuser lab showed that the separation of the two kinetochore clusters is not a reliable read-out for bi-orientation - rather the CENs need to be labeled and tracked (Marco et al, 2013). Given that the conclusion reached from this assay (kinetochores bi-oriented faster and correctly) becomes an assumption for almost all subsequent experiments, the authors should confirm their results using a more high-resolution assay.

A separate point here is that a predominant phenotype in Figure 1D (and 4B) appears to be the increased spread/scatter of the kinetochore clusters, which may be indicative of faulty bi-orientation or some other defect. This is an important point - since, as mentioned above, the authors use the conclusion that the basic patch mutant results in "better" error correction as assumptions for the remaining experiments in the paper.

And finally in Figure 1D, the authors measure an increase in kinetochore-kinetochore distance. They conclude that expression of the binding patch mutant results in "enhanced force generation" (stronger kinetochore - microtubule attachments). This cannot necessarily be concluded from the k-k distance data alone. Just as an example, wouldn't a similar phenotype be observed in the case of defects in the cohesin pathway or in mis-regulation of chromatin organization? In addition, the conclusion that kinetochore-microtubule attachments are stronger (generate more force) is not consistent with their model that recruitment of PP1 results in stable kinetochore-MT attachments while reduced recruitment promotes kinetochore-MT turnover ('error correction'). How do the authors explain how a decrease in PP1 might increase kinetochore-MT forces?

(5) In figure 2C, the authors report that cells expressing the binding patch mutant have a slight delay in transitioning from metaphase to anaphase. They suggest that the delay is a result of reduced Glc7 recruitment and therefore increased pMELTs and SAC protein retention. They use this as further evidence that the binding patch mutant recruits Glc7. Two questions: (1) is this difference significant? (2) are there other explanations for the slight delay (e.g. defects in chromosome biorientation as observed in Figure 1D?)

(6) In Figure 3, the authors show that the Spc105 binding patch mutant rescues lethality of a Sgo1-deletion. Sgo1 is involved in the "error correction" pathway (by recruiting the CPC) and its loss would presumably result in more stable kinetochore-microtubule attachments and a decrease in error correction efficiency. Because the binding patch mutant rescues Sgo1Δ, the authors use this as further evidence that the binding patch recruits Glc7, with the rationale being that the binding patch mutant recruits less Glc7, resulting in higher kinetochore-microtubule turnover, which in turn rescues cells from the proposed hyper-stable kinetochore-microtubules in sgo1ΔΔcells. Given the role of the basic patch in binding microtubules, an alternative explanation is that the loss of Spc105-mediated microtubule binding in cells expressing the basic patch mutant rescues the hyper-stable attachments in the Sgo1Δ cells.

(7) In Figure 4E, what is the rationale for testing chromosome alignment/error correction in cells expressing the Spc105-RASA mutant in the Sgo1Δ cells? As an extra challenge to error correction? Is the effect still observed in wild-type cells? Also, in the graph shown in figure 4E, bottom left, are the differences between WT and mutant Spc105 significant at the two time points?

(8) The authors demonstrate that premature recruitment of Glc7 to the N-terminus of Spc105 in early mitosis (using an FRB-FKBP approach) results in defects in chromosome alignment/biorientation (Figure 5). This provides evidence that Ipl1 needs to phosphorylate Spc105 to prevent premature loading of Glc7 and likely premature stabilization of kinetochore-MT attachments. In the Rosenberg, 2011 study, the authors use an Spc105 RVAF mutant for the same ends - to constitutively recruit Glc7 to kinetochores. In this case, the authors reported no defects in cell cycle progression or chromosome segregation. Have the authors here tested a similar mutant? Is it possible that fusing Glc7-FKBP to FRB-fused Spc105 causes unintended problems in early mitosis? They show that these modules/fusions don't affect chromosome segregation when targeted in late mitosis after biorientation has occured, but it could be that these fusions only cause issues during the process of generating attachments. It seems the RVAF mutant might be a more straightforward way to address this, as it should also prematurely recruit Glc7 to kinetochores.

Additional comments:

1. The authors conclude that the basic patch / MT binding domain (in addition to the RVSF motif) in Spc105 is important for Glc7 recruitment in budding yeast. This is somewhat different than the case in C. elegans, where the two domains have distinct and non-overlapping activities (Espeut et al., 2012) This potential difference across systems is worth mentioning. Related to this, it is probably also important to note that in C. elegans, the KNL1 MTBD is also involved in forming load-bearing kinetochore-MT attachments, suggesting that the roles of KNL1-recruited may be different across species.

2. In a couple of instances, the phrase "error correction" is used, but I think the authors mean "attachment stabilization." For example: Page 12, bottom: "We find that Glc7 activity required for error correction and SAC silencing is derived from independent sources." Glc7's "activity" is kinetochore-MT stabilization, whereas its absence (according to the authors) is required for kinetochore-MT turnover or "error correction." [same for Page 12, bottom, "The source of Glc7 activity used in error correction is unclear."]

3. A recent paper (Suzuki et al., 2018) reported that in budding yeast, Cin8 recruits a population of PP1/Glc7 to kinetochores specifically in late mitosis to generate tension (increase binding) between Ndc80 complexes and microtubules. This suggests a recruitment mechanism for the "other" population of PP1 that the authors here propose, but do not identify. This relevant study should be mentioned.

4. As rationale to explore the Spc105 basic patch as a potential recruiter of Glc7, the authors state, "Finally, basic patch activity can be disrupted by phosphorylation of the Serine and Threonine residues immediately downstream." Is this from previously published work? A citation or Figure callout would be helpful.

5. In the main text or in Figure 1, it would be useful to state how the basic patch residues RRRK were mutated to generate the basic patch mutant.

6. Page 4: What is meant by "single molecule microscopy?" Are we looking at single molecules in Figure 1? My interpretation from the text is that many molecules are coated on a bead.

7. Page 4: What is meant by "recombinant phospho-domains?" The N-terminal region, the MELT motifs, all of them together?

8. Figure 1E: The authors state in the text that Mad1 is not detectable (and not changed) in WT or mutant cells. By my eye, it looks increased. It would be helpful to see a quantification of Mad2 in control, early prometaphase cells or nocodazole-treated, so that the reader has a reference of what maximally high levels would be. The same could be said for Bub3.

9 Page 7: The authors state that "Higher Sgo1 recruitment will in turn enhance sister centromere cohesion and Aurora B activity thus resulting in better biorientation and error correction." More Aurora doesn't necessarily mean "better" biorientation and correction - sometimes means worse.

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

- *"In their manuscript titled "Minimization of cross-talk between Spindle Assembly Checkpoint silencing and error correction", authors Roy, Joglekar and colleagues investigate the functions of the N-terminal tail of Spc105/Knl1 in chromosome segregation using budding yeast as a model system. They examine two previously uncharacterized regions of the budding yeast Spc105 that are rich in basic residues. Mutants in these basic patches have phenotypes reminiscent of a milder version of mutants in this region that disrupt phosphatase binding, resulting in a metaphase delay due to decreased silencing of the spindle assembly checkpoint. The authors then go on to show that these mutants are able to suppress the phenotype of mutants in the chromosome biorientation pathway. Furthermore, artificially targeting of the phosphatase to Spc105 during the error correction process decreases the rate of chromosome biorientation.*

  Together, these results suggest two key advances. Firstly, that PP1 recruitment to Spc105 affects chromosome biorientation in a manner independently of the two known pathways: checkpoint silencing and Sgo1 recruitment to the inner centromere. Secondly, that this activity must be regulated to prevent premature stabilization of incorrect attachments.

  However, it is not clear to me that any of the phenotypes shown are independent of this known checkpoint-silencing function of PP1-Spc105. It has been previously demonstrated that increased time in metaphase will increase the accuracy of chromosome segregation by giving the chromosomes more time to make proper attachments (nicely demonstrated in Munoz-Barrera 2015 for example). Unless the authors can convincingly demonstrate that the phenotypes they observe are independent of the known functions of Spc105-recruited PP1, I recommend that this paper be published in journal with less emphasis on novelty."

Author's response: All the major concerns expressed by the reviewer were justified. We performed several new experiments to address all the concerns raised. We demonstrate that:

(1) chromosome biorientation is faster in cells expressing Spc105^{BPM} by visualizing single centromeres using TetO-TetR-GFP.

(2) Spc105^{BPM} significantly reduces PP1 association with kinetochore particles (~60-80%) using kinetochore particle pull-downs

(3) mutation of the other known PP1 binding site, the GILK motif, causes milder, but similar and additive phenotype as Spc105^{BPM}

(4) the improved chromosome segregation is not due to delayed SAC silencing (spc105^{RASA} partially rescues bub3Δ, rescues mad2Δ sgo1Δ as well as mad2Δ bub1$^{\Delta kinase}$

(5) the phenotypes observed in the Glc7-tethering experiment (prior to chromosome biorientation) are not due to early anaphase onset by blocking anaphase onset in these cells.

These and other results greatly strengthen our main conclusion: weakening of the Spc105-PP1 interaction during prophase is crucial to ensure that syntelic attachments are resolved prior to SAC silencing and anaphase onset. Our detailed response is included below.

- *"Major concerns:*

  1. To elaborate on the main point above, the phenotypes observed for the basic patch mutants include increased localization of the checkpoint proteins Bub3 and Mad1 (Fig. 1E), a cell-cycle delay (Fig. 2C), and rescue of mutants in the biorientation pathway (Fig. 3B and 4E). All of these phenotypes can be explained by a delay in metaphase due to slower SAC silencing.

  The experiment that strongly argues against this interpretation is the rescue of sgo1D benomyl sensitivity with a RASA mad2D triple mutant. Unfortunately for the two strains shown, one of them showed rescue and the other one did not, making interpretation of this result rather murky. A clear demonstration of sgo1D rescue on benomyl plates independent of the checkpoint for both the RASA and BPM mutants would go a long way to support the claims of a more direct role in chromosome biorientation."

Author's response: We understand this concern. We should have explained the rationale behind showing the two biological replicates. Typically, *sgo1*Δ mutants show strain-to-strain variability in growth rates (this can also be seen in another experiment involving sgo1Δ in Fig. 5D). The slow-growing segregant appears to not grow on benomyl after a 3-day incubation that we used for the rest of the experiment. However, a closer inspection will reveal slow growing colonies.

To clearly demonstrate this phenotype, we repeated the spotting assay with three different segregants and incubated the benomyl 30µg/ml (ben^{30}) plate at 30°C for 5 days instead of three. The difference of growth among the three biological replicates is visible in YPD. However, the additional time provided for colony growth clearly shows the suppression of benomyl lethality.

The new data are shown as a Fig. 4F in page 28.

Additionally, to get around the issue of variable growth rates of *sgo1Δ* transformants, we created a triple mutant: $spc105^{RASA}$, $bub1^{\Delta kinase}$, *mad2Δ*, which also has impaired Sgo1 recruitment and a non-functional SAC. $bub1^{\Delta kinase}$, *mad2Δ* does not grow on benomyl, but the triple mutant grows robustly.

This result is also displayed in Fig. 4F on page 28.

- *"I would recommend combining the BP and RASA mutants with the spc105-6A mutant that is used as a control in the benomyl-sensitivity experiments. This should demonstrate that the rescue is independent of both the checkpoint silencing and Sgo1-recruitment pathways.*"

Author's response: Following the logic suggested by the reviewer, we now show that $spc105^{RASA}$ partially suppresses the benomyl lethality due to *bub3Δ*. $spc105^{BPM}$ also results in a weaker suppression of the benomyl lethality of *bub3Δ*. The difference between the two strains is likely $spc105^{BPM}$ reduces the recruitment of PP1 by ~ 62-85%, whereas $spc105^{RASA}$ eliminates this recruitment. This experiment is analogous to the *spc105-6A* test suggested by the reviewer.

This result is displayed in Fig. 4E on page 28.

- *"The fact that the RASA and BPM mutants very clearly show rescue of the mad2D on benomyl plates does indicate some role in chromosome segregation outside of the checkpoint function, which is quite interesting. However, this additional role is not necessarily in error correction."*

Author's response: This concern is mainly the result of a confusing description of the results in the original manuscript. We are not suggesting that Spc105BPM directly improves error correction. Rather, the RASA and BPM mutations improve chromosome biorientation by minimizing/reducing inadvertent dephosphorylation of microtubule-binding kinetochore proteins and thus stabilizing any syntelic attachments that form initially. The most direct evidence for this comes from the suppression of benomyl lethality of *ndc80-6A dam1-3A* double mutant by *spc105^{BPM}* (Fig. 3).

We have now revised the manuscript extensively to clearly explain this point as follows:

Page 10-11: "A weakened interaction between Glc7 and PP1 may also improve kinetochore biorientation simply by delaying SAC silencing, and thus providing additional time for kinetochore biorientation (Munoz-Barrera et al., 2015). To test this notion, we exploited the SAC-deficient *spc105^{RASA}* strains and the benomyl sensitivity assay as a functional test of the accuracy of chromosome segregation. Due to the inactive SAC in these strains (Fig. S2E), any improvement in the accuracy of chromosome segregation must occur due to improved sister kinetochore biorientation and error correction. Strikingly, *spc105^{RASA} mad2Δ* grew robustly on benomyl-containing media, even though *mad2Δ* grew poorly under the same condition (Fig. 4E). *spc105^{RASA}* also partially suppressed the benomyl lethality of *bub3Δ,* the milder rescue likely reflecting the additional defects due to *bub3Δ* that include lower Sgo1 recruitment to the centromere and defects in APC/C function [Figure 4E, also see Fig. S2F, (Yang et al., 2015)]. Consistent with these results, *spc105^{BPM}* suppressed the benomyl sensitivity due to *mad2Δ*, and mildly suppressed the benomyl lethality due to *bub3Δ*. Most strikingly, the triple mutants *spc105^{RASA} mad2Δ bub1^{Δkinase} or spc105^{RASA} mad2Δ sgo1Δ* also grew on benomyl-containing media (Fig. 4F). Thus, the improved accuracy of chromosome segregation observed upon the weakening or loss of the Glc7-Spc105 interaction is not because of delayed SAC silencing."

- *"I believe that the checkpoint-silencing role could also explain the biorientation phenotype in Figure 5A. Too much phosphatase activity could prevent checkpoint activation and allow premature progression into anaphase with misattachments. I could not find any mention that this experiment was conducted in a metaphase arrest condition such as cdc20 depletion, which would help alleviate this concern. Premature progression into anaphase could also explain the increase in spindle length.*"

Author's response: This is a valid concern. We only partially addressed it by showing that the recruitment of Bub3 to unattached kinetochores remains unaffected despite the tethering of Glc7 (Fig. S3A). However, to fully ensure that the spindle elongation observed in this assay is not due to an early anaphase onset, we blocked anaphase in this experiment by repressing *CDC20* as suggested by the reviewer. The results of this new assay were nearly identical to the old one. This confirms that the spindle elongation is indicative of weak inward force generated by the fewer bioriented chromosomes rather than an early anaphase onset.

The new result in shown in Fig. 5A on page 30.

- *“2. I don't see how the "kinetochore biorientation" assay used in figures 1D and 4B is measuring biorientation. This assay is looking as all of the kinetochores at once, such that chromosomes that have not yet bioriented would not be readily discernable from those that have bioriented. Measuring the timing of biorientation would require observing individual kinetochores, or in extreme cases, looking as kinetochore asymmetry as is shown in figure 5A.”*

Author’s response: We used centromere-proximal TetO repeats to observe the kinetics of biorientation for a single chromosome. This experiment confirms that individual chromosomes achieve biorientation faster in cells expressing Spc105^{BPM}.

This result is displayed in Fig. 1C on page 24.

- *“On a similar note, the authors claim that the Bub3 and Mad1 localization are on "bioriented kinetochores" and that the cells are "in metaphase". I see no indication of cell cycle synchronization, so how do the authors know that all of the kinetochores on these spindles are already bioriented and that the chromosomes are not still in the process of error correction?”*

Author’s response: We are sorry for this omission. We conducted these experiments after depleting Cdc20 to block the onset of anaphase.

This is clearly mentioned in the text as follows and also highlighted in Fig. 1E on page 24.

- *“3. The in vitro data strongly suggests that mutations in the basic patch strongly affect microtubule binding directly and have no measurable effect on PP1 binding. I found it very surprising that the authors conclude from this data that "Any potential microtubule-binding activity of the basic patch is unlikely to be important for the phenotypes discussed here." It seems that a model where the microtubule-binding activity of the basic patch(es) somehow acts together with the phosphatase-binding activity of the RVSF motif would be more in line with the presented data.*”

Author’s response: This was a weak point of the submitted manuscript. As mentioned earlier, we conducted “kinetochore particle pull-downs” following the methodology established by the Biggins lab. Briefly, we immunoprecipitated Dsn1-HIS-Flag from yeast cell extracts, and then quantified the amount of Spc105-mCherry (either wild-type or with the basic patch mutation) and Glc7-3xGFP that co-precipitates. In two repeats of this experiment, we found ~62-85% reduction in the amount of Glc7 coprecipitating with the kinetochore particles.

We also found that the fusion of the TOG2 domain from Stu2/XMAP215 to Spc105^{BPM}, which is a bona fide microtubule-binding domain, does not suppress the abnormal Bub3 recruitment phenotype caused by the basic patch mutation. This experiment further suggests that microtubule-binding activity is not sufficient to explain the role of the basic patch. Nevertheless, without knowing the exact mechanism, we cannot rule out that the ability of the basic patch to bind to the microtubule somehow contributes to PP1 recruitment.

- *“Minor concerns:*

1. The authors state that "However, this inhibition of PP1 recruitment by Aurora B is mainly thought enhance the recruitment of SAC proteins, and contribute to robust SAC signaling (Nijenhuis et al., 2014). The possibility that this inhibition is crucial for efficient error correction has not been considered." To say that something has not been considered is strange, and it didn't take me long to find this in the literature: "However, these data may still be consistent with a role for both PP1-Knl1 and PP2A-B56 in antagonizing Aurora B, since the negative effects of PP1-Knl1 mutation could simply be masked by a compensatory increase in kinetochore PP2A-B56 (as a result of phosphatase cross-talk; see Figure 3H). In agreement with this hypothesis, rescuing PP1-Knl1 following PP2A-B56 depletion is sufficient to reduce Aurora B activity and improve chromosome alignment (Nijenhuis et al., 2014)." from a review by Adrian Saurin this year. This sentence should be rephrased."

Author's response: Our intention behind writing that statement was to point out specifically that the possibility that the SAC silencing *mechanism can inadvertently stabilize syntelic attachments* has not been considered. Instead of facilitating two different mechanisms, this cross-talk in two functions will be harmful.
In the Saurin review, the author mainly discusses the relative importance of PP1-Knl1 versus PP2A-B56-Knl1 to the stabilization of kinetochore microtubule attachments in the context of promoting chromosome biorientation. *It does not discuss the possibility that PP1-Knl1 can stabilize syntelic attachments creating a situation harmful to chromosome segregation.*

To avoid the appearance of discounting prior work, we rephrase this statement as follows:

Last paragraph on page 3: "PP1 recruited by Spc105 is necessary for SAC silencing, and the same PP1 is also thought to contribute to stabilization of kinetochore-microtubule attachments (Hendrickx et al., 2009; Liu et al., 2010; London et al., 2012; Nijenhuis et al., 2014; Rosenberg et al., 2011). Interestingly, Aurora B, the kinase responsible for error correction, downregulates Spc105-PP1 interaction, but this is mainly thought to be important for robust SAC (Liu et al., 2010; Nijenhuis et al., 2014). Using a combination of cell biological and genetic experimentation, we show that the down-regulation of PP1 recruitment by Spc105 is critical for chromosome biorientation and accurate chromosome segregation."

- *"2. Labeling of the Ndc80 complex is typically done through Nuf2 (Joglekar et al 2006, Joglekar et al 2008). Is this because labeling Ndc80 directly leads to a chimeric protein that is not fully functional? It seems possible that Glc7 targeting to Ndc80 does not affect chromosome segregation because the tagged Ndc80 is being outcompeted for kinetochore occupancy by the wild-type."*

Author's response: Ndc80-GFP is functional, and we have used it in many different studies in addition to the ones cited above. The chimeric protein is recruited to the kinetochore at the same level as any of the other Ndc80 components including Nuf2-GFP. Nonetheless, we decided to take this experiment out of the manuscript, because it is only peripherally related to the main findings and the model synthesized in the study.

- *"3. The example images in Fig. 4E are very difficult to interpret. The weak contrast and merging of the two colors make the centromere dots very hard to see."*

Author's response: We have changed the contrast to ensure that the micrographs are clearly visible.

- *"4. The authors state that "Spc105BPM improves the accuracy of chromosome segregation". This implies that accuracy is somehow improved under normal/all conditions, not that is suppresses the segregation errors caused by a specific mutation (sgo1D). "*

Author's response: Observing an improvement in chromosome segregation in budding yeast under normal conditions is quite difficult because segregation errors are rare. Therefore, almost all of the assays used here rely on challenging the cells in some manner (benomyl sensitivity, sgo1Δ). As this reviewer mentioned earlier, chromosome segregation accuracy is improved if cells are afforded more time to biorient their chromosomes. Conversely, if chromosomes achieve biorientation at a faster rate, the effect will be more accurate chromosome segregation. We have not confirmed this implication.

Therefore, we revised the sentence as follows in page 9: Spc105^{BPM} improves the accuracy of chromosome segregation in sgo1Δ cells in page 9.

- *"5. The authors refer to the Spc105D80-128 mutant as "the basic patch and surrounding residues", however this deletion starts directly after the RVSF PP1-binding motif. Based on the fact that the basic patch has no effect on PP1 binding in vitro but the RVSF motif does, it seems more likely that this is a partial disruption of the RVSF-binding function, not an increased disruption of the basic patch. I would describe this mutant as "basic patch plus RVSF-adjacent."*

Author's response: We agree with this. However, because we have much more direct evidence for the involvement of the basic patch in recruiting Glc7 in the form of kinetochore particle pull-down experiments, we have decided to remove this confusing experiment from the revised manuscript.

- *"6. The graph in figure 2C measures the time from G1 to anaphase, yet the manuscript states that it measures "metaphase-to-anaphase"."*

Author's response: We have corrected this oversight.

- *"7. The authors state that "Thus, spc105BPM acts as a gain of function mutation by reducing Glc7 recruitment to the kinetochore via Spc105." This statement is not consistent with the in vitro binding data."*

Author's response: We have removed this statement.

- *"8. I am very confused by these two juxtaposed statements: "The stabilization of bipolar attachments is achieved by an Spc105-independent Glc7 activity. Second, our data imply that Glc7 recruited by the RVSF motif interferes with error correction" These two concepts seem contradictory. Is this implying that Glc7-Spc105 interferes with error*

correction in a mechanism other that stabilizing MT-KT connections? Are the authors trying to say that Glc7-Spc105 can stabilize connections, but ordinarily doesn't?"

"Overall, the manuscript would greatly benefit from more clarity in the writing."

Author's response: We have made significant revisions to the entire manuscript in addition to including the results of new experiments. The specific statement mentioned above has been changed as follows:

Page 11: **"**Thus, Glc7 recruited by the RVSF motif of Spc105 is not required for stabilizing kinetochore-microtubule attachments. On the contrary, our results imply that the Glc7 recruited by the RVSF motif interferes with the formation of bipolar attachments and error correction, likely by stabilizing syntelic kinetochore-microtubule attachments and by interfering the error correction mechanisms. When the RVSF motif is inactivated, these deleterious effects are eliminated…"

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

- *"The KNL1 family of kinetochore proteins play a central role in co-ordinating chromosome bi-orientation and spindle checkpoint (SAC) signalling. Members of the KNL1 family contain binding sites for microtubules, type-1-phosphatase (PP1) and components of the SAC machinery (particularly Bub3-Bub1). In this study Roy and colleagues propose that a basic patch motif (BPM), located at the N-terminal region of Spc105 (budding yeast homologue of KNL1), binds both microtubules and aids the recruitment of Glc7. Secondly, they propose that delayed interaction of Glc7 with Spc105 ensures chromosome bi-orientation before SAC silencing. Unfortunately, the experimental logic and presented evidence do not support the conclusions of the manuscript."*

  Major issues.

  A previous study in C.Elegans identified a microtubule binding site in KNl1 immediately N-terminal, but distinct from, the G/SILK PP1-binding motif (Espeut et al., 2012. J. Cell Biol. 196, 469). Inspired by this study the authors show that mutation of four basic residues RRRK (a.a. 101-104) in budding Spc105 (Spc105-BPM) inhibits interaction with microtubules in vitro (Figure 1C). This is convincing. Mutation of the basic patch does not, however, influence interaction with Glc7 (PP1) (Text page 6; Figure S1C). As such it would seem that the microtubule binding and PP1 binding sites in Spc105 can be separated, consistent with evidence from C.Elegans.

  However, the authors argue that the basic patch does influence binding to PP1 by showing that deletion of a larger region of Spc105 (D80-120) displays reduced PP1 binding (Figure 1C). Unfortunately, recent structural data reveals that a hydrophobic region immediately C-terminal to the conserved RRVSF motif of human KNL1 interacts with the catalytic subunit of PP1 (Bajaj et al., 2018, Structure 26, 1327). Since the Spc105(D80-120) mutant removes sequences immediately C-terminal to the RRVSF motif, the conclusion that the basic patch contributes to binding of PP1 to Spc105 is not supported. This undermines the interpretation and conclusion of experiments in the rest of the paper."

Author's response: We understand this concern. We want to point out that there are two separate conclusions here. (1) Mutation of the basic patch in Spc105 reduces its ability to recruit PP1. (2) Reduced PP1 recruitment via Spc105 improves chromosome biorientation and segregation.

The reviewer is quite correct to point out that our *in vitro* pull-down experiment using a recombinant phosphodomain was inconclusive in answering the first question. We used kinetochore particle pull-down to confirm that mutation of the basic patch reduces PP1 recruitment to the kinetochore by ~ 62-85%. The second question is whether reduced PP1 interaction with Spc105 improves chromosome biorientation. We have presented ample data to support this conclusion, including analysis of genetic interactions of $Spc105^{RASA}$. We even show that artificial tethering of Glc7 in prophase, but not metaphase, leads to chromosome biorientation defects.

We agree with the reviewer's concern that $Spc105^{\Delta 80\text{-}128}$ mutation may affect the activity of the RVSF motif as well. Our main intention behind using this mutation was to show that a weakened Spc105-PP1 interaction causes the same phenotype as $Spc105^{BPM}$. Following the suggestion by this reviewer, we conducted a detailed analysis of the $Spc105^{GILK:AAAA}$ mutant to show that it phenocopies $Spc105^{BPM}$, but produces a milder effects.

- *"Indeed, it is hard to rationalise why mutation of a region of Spc105 that is involved in binding PP1 would lead to increased resistance to microtubule destabilising agents and improve the efficiency of chromosome bi-orientation. One would expect outer kinetochore proteins to be more highly phosphorylated (less phosphatase bound) leading to more unstable kinetochore-microtubule interactions. Bajaj and colleagues recently shown that the interaction of PP1 and microtubules with human KNL1 is mutually exclusive (Bajaj et al., 2018, Structure 26, 1327). If this competition occurs in vivo then the Spc105-BPM may infact bind more PP1 leading to stabilisation of microtubule-kinetochore interactions. Regardless, a tangible molecular explanation for the effect of the spc105-BPM mutant on microtubule stability and chromosome bi-orientation is currently missing.*"

Author's response: This is precisely our point! The results discussed here are hard to rationalize using the accepted notions regarding the phosphoregulation of kinetochore proteins and the effects of PP1 on sister kinetochore biorientation. Instead, our extensive data demonstrate that the weakening/elimination of Spc105-PP1 interaction results in more efficient kinetochore biorientation and more accurate chromosome segregation. Our original submission failed to clearly articulate this proposed mechanism behind improved sister kinetochore biorientation.

The *in vitro* results by Bajaj et al. are clear. They show that the phosphodomain of Spc105 can bind to either PP1 or the microtubule, but not both at the same time. However, what is not clear is how these findings apply to the *in vivo* operation of the kinetochore. Unattached kinetochores, which should enjoy unimpeded PP1 interaction, do not require PP1 activity. According to Bajaj et al. kinetochore-microtubule attachment should inhibit PP1 recruitment, but this is when PP1 activity is needed to silence the SAC and to stabilize attachments. Bajaj et al claim that the stronger binding PP1 will outcompete microtubules. What then is the purpose of the microtubule binding by Knl1 in the first place? It is clearly not required for force generation as shown by the Desai lab in C. elegans and also the Nilsson lab in human cells. Furthermore, results from HeLa cells show PP1 recruitment peaks in metaphase, after kinetochore biorientation has been achieved (Liu et al 2009).

Our model proposes an alternative role for the basic patch, and clearly demonstrates the necessity of regulated PP1-Spc105 interaction in chromosome biorientation. This conclusion is based on the following line of evidence:

1. Using kinetochore-particle pull-down experiments, we show that the mutation of the basic patch significantly weakens PP1 recruitment to the kinetochore (Fig. 2A and Fig. S1E).
2. In Fig. 1, we observe the biorientation of one pair of sister centromeres to find that the mutation of the basic patch leads to faster kinetochore biorientation (Fig. 1C).
3. In Fig. 3, we provide extensive data showing that *spc105^{BPM}* suppresses the benomyl lethality of strains carrying mutations in nearly every major factor involved in chromosome biorientation (e.g. *sgo1Δ, bub1^{Δkinase}*, *rts1Δ*, *ipl1-2*, *ndc80-6A dam1-3A*). The latter observations are especially crucial. They indicate that when Glc7-Spc105 interaction is weakened, chromosome biorientation can be ensured even with sub-physiological levels of Aurora B kinase activity (*ipl1-2)* or when its substrates have only one regulatable residue (*ndc80-6A, dam1-3A)*. In other words, sister kinetochore biorientation is fundamentally improved. This translates into a higher chromosome segregation accuracy (Fig. 4A).
4. Improved chromosome segregation is not the result of delayed SAC silencing, because *spc105^{RASA}* rescues benomyl lethality of *mad2Δ* and *bub3Δ* mutants (Fig. 4E).

5. Finally, we use artificial tethering of Glc7 to Spc105 to demonstrate that a premature recruitment of Glc7 in prophase-like conditions interferes with sister kinetochore biorientation (Fig. 5A).

We clearly describe the overall model in the revised discussion on Pages 13-14.

- *"The authors should note that both the S/GILK and RRVSF motifs in human KNL1 and fission yeast Spc7 contribute to association of PP1 in vitro, although the interaction of PP1 with the S/GILK motif is weaker than with the RRVSF motif (Meadows et al., 2011, Developmental Cell 20, 739; Bajaj et al., 2018, Structure 26, 1327). Mutation or deletion of the RRVSF motif in either budding yeast Spc105 or fission yeast Spc7 causes cell cycle arrest due to hyperactivation of the SAC; namely the lethality is rescued by deletion of Mad2 or Mad3, components of MCC. This suggests that interaction of PP1 with the RRVSF silences the SAC (Rosenberg et al., 2011, 21, 942; Meadows et al., 2011, Developmental Cell 20, 739). However, the GILK motif in Spc7 is also essential for viability in fission yeast but this is not bypassed by inactivation of Mad2 or Mad3 (Meadows et al., 2011, Developmental Cell 20, 739). Since in the authors hands Glc7 (PP1) binds weakly to the Spc105(RASA) mutant (Figure S1C) the authors may like to consider whether the GILK motif in Spc105 contributes to PP1 binding and whether this association is more important for chromosome bi-orientation than for SAC signalling. Differential functions, regulation and timing for the association of PP1 to the G/SILK and RRVSF motifs, respectively, may provide a more compelling explanation as to how Spc105-PP1 co-ordinates chromosome bi-orientation and SAC silencing."*

Author's response: This was a great suggestion. We have now analyzed the importance and activity of the GILK motif in Spc105. Unlike fission yeast, this motif is not essential for cell survival. However, consistent with reduced PP1 recruitment: we find that $Spc105^{GILK::AAAA}$ leads to higher fraction of metaphase cells recruiting Bub3-Bub1. This effect is weaker than, but similar to, the effect of $Spc105^{BPM}$. In fact, the effect appears to be additive: a mutant Spc105 wherein both the basic patch and the GILK motif has been mutated has an even higher fraction of cells recruiting Bub1 in metaphase (Fig. 4B). Moreover, we observed that $Spc105^{GILK:AAAA}$ is not able to suppress the benomyl sensitivity of bub1-ΔK (Fig. 4C).

The new data are displayed in Fig. 4 and discussed on page 10.

- *"Other issues*
  The authors suggest that phosphorylation of serine and threonine residues to the C-terminus of the basic patch might influence microtubule and/or PP1 binding (Figure 4F). This referee could find no data showing that these residues are phosphorylated in the Craig et al., 2004 paper or in other phospho-proteome databases (e.g. https://thebiogrid.org/33158/protein). Direct evidence should be presented that these residues are phosphorylated during mitosis or the mutant data should be removed."

Author's response: We apologize for the oversight. We included that citation as a reference to the original publication that described the Global Proteome Machine Database. This database hosts a public repository of all the quantitative mass spectrometry observations made by the community. The observations specific to Spc105, which show the residues in question as phosphorylated in at least two different studies can be accessed here:

http://psyt.thegpm.org/~/dblist_pep_modmass/label=YGL093W&modmass=80@STY&display=0

This url is included in the Methods section on Page 18. Additionally, we cite the two studies that generated the mass spectrometry data on Page 4.

- *"It is not terribly surprising that mutation of the basic patch does not further increase Bub3 levels at the kinetochore in the presence of nocodazole (Figure 2A) as there would be no microtubules present and Mps1 would be maximally active."*

Author's response: As pointed out in our manuscript, we have previously shown that inhibition of PP1 does not have any effect on the recruitment of SAC proteins to the kinetochore (Aravamudhan et al. 2016). It is equally important to note that loss of PP1 recruitment leads to increased Bub3-Bub1 binding to human kinetochores even when they are unattached and have maximal Mps1 activity (Zhang et al. EMBO Journal).

We include these citations in two different contexts: **on Page 7 to make above point**, and **again on Page 14 to point out that the deletion of the basic patch in human cells does not affect chromosome biorientation**.

- *"The title of Figure legend 2 is incorrect. This figure shows no data on error correction."*

Author's response: We apologize for this oversight. It has been corrected.

- *"The recent paper from Page and colleagues (Bajaj et al., (2018) Structure 26, 1327-1336) detailing the structure of the KNL1-PP1 complex should be properly discussed and referenced.*

Author's response: This paper is discussed as follows:

Page 14: "A recent *in vitro* study found that Knl1 binding to the microtubule and PP1 is mutually exclusive, and proposed the model wherein microtubule-binding contributes to regulated PP1 binding to KNL1 (Bajaj et al., 2018). However, this model does not explain why microtubule-binding by KNL1 is needed in the first place if: (a) PP1 binding is inhibited by phosphorylation in unattached kinetochores, where microtubules are absent, and (b) the stronger binding PP1 is expected to displace the microtubule from KNL1 anyway, even when microtubules are present. The true functional significance of the microtubule-binding activity of basic patch in KNL1, which does not contribute to kinetochore force generation, needs to be fully understood."

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

- "*In this manuscript (Minimization of cross-talk between Spindle Assembly Checkpoint silencing and error correction), the authors investigate kinetochore functions of KNL1/Spc105-recruited PP1/Glc7 in budding yeast. The large kinetochore scaffold protein Spc105 (KNL1 in human cells) recruits the phosphatase PP1/Glc7 to kinetochores via its N-terminal RVSF motif. In many organismal systems, it has been demonstrated that PP1 is in involved in both stabilizing kinetochore-microtubule attachments and silencing the SAC. Because cells need to silence the SAC only after kinetochore-microtubules are correctly formed and stabilized, the authors here reason that these two functions of PP1 need to be carried out sequentially. To investigate how this might be facilitated the authors test if Spc105-recruited Glc7 has a role in kinetochore-microtubule attachment regulation. The major conclusions they draw are: (1) Spc105 recruits Glc7 for SAC silencing but not kinetochore-MT attachment regulation and therefore, there must be an Spc105-independent pool of Glc7 that functions to regulate kinetochore-MT attachments; and (2) kinetochores must delay Glc7 recruitment until correct attachments are made so that the SAC is not pre-maturely silenced. The issue here is an important one: if there is only mode of recruitment for Glc7, attachment security and SAC silencing would occur simultaneously rather than sequentially. Thus, a second Glc7/PP1 pool is likely required for stabilization of attachments and is recruited to the kinetochore-MT interface based on criteria that are different than those required for recruitment to Spc105 and SAC silencing.*

  I think the authors have developed an important new model for PP1/Glc7 recruitment and function at kinetochores. However, many of the results are redundant with past findings (Rosenberg et al., 2011; London et al., 2012; Nijenhuis et al., 2014; Espeut et al., 2012; Liu et al., 2010). What is actually new here is the re-framing and evaluation of the results in the context of this new model. With that being said, there is some new information provided, and the study should be of interest to the field. However, there are some concerns that preclude recommendation of the manuscript for publication in the JCB in its current form. For example, the first third of the paper uses a mutant of Spc105 as a tool to reduce PP1/Glc7 levels at kinetochores, however, it is not convincing that this mutant has any role in Glc7 recruitment. In addition, a major premise of the study is that reduction of PP1/Glc7 at kinetochores results in "faster" kinetochore bi-orientation, which is used a base assumption for much of the paper. As presented, the data do not necessarily support this conclusion. These and other issues that should be addressed are described in detail below.

Author's response: We thank this reviewer for their in-depth comments. The comments helped us in significantly improving the manuscript. We agree with the assessment that, with the exception of the involvement of the basic patch in recruiting PP1, we have not identified any new activity or protein. However, our work has uncovered a crucial and overlooked aspect of kinetochore regulation: a harmful cross-talk between SAC silencing and chromosome biorientation/error correction that arises because of the involvement of PP1 in both functions. Our work explains how this cross-talk is mitigated in budding yeast, and thus synthesizes a new understanding of kinetochore function *in vivo*. These findings also cast existing data from human cells into a new light.

- "*Major comments:*
  (1) The authors conclude early on that the Spc105 basic patch has an important role in recruiting Glc7 to kinetochores, but the data supporting this conclusion are not convincing. Clearly the basic patch binds MTs - this has been shown in other systems and the authors confirm those findings nicely here. But the evidence demonstrating that this motif recruits Glc7 is not compelling. For example: The authors carry out an experiment (shown in Supplemental figure 1C) to "directly test whether the basic patch contributes to Glc7 recruitment by Spc105." Here, they quantify the amount of Myc-Glc7 that is pulled out of yeast lysates with beads coated with various Spc105 fragments. While they show clearly that Spc105-RASA (a mutant that cannot bind Glc7) is impaired for Glc7 binding, Spc105-BPM is not. This seems to be clear evidence that the basic patch does not function to recruit Glc7 to Spc105."

Author's response: This was a major weakness of the submitted manuscript. Using the kinetochore particle pull-down methodology established by the Biggins lab, we now show that kinetochore particles containing $Spc105^{BPM}$ recruit significantly lower amount of PP1 than kinetochore particles with wild-type Spc105 (Figure 2A). We observed reduction of Glc7 level by 62-85% in two independent experiments. The mechanism by which the basic patch contributes to PP1 recruitment is unclear.

Kinetochore particle pull-down experiments shown in Fig. 2A and Fig. S1E show that the loss of the basic patch results in a significant reduction in the amount of PP1 co-precipitating with kinetochore particles.

- *"In this experiment, the authors also use an Spc105 mutant deleted for amino acids 80-128, which removes the basic patch and an additional 45 amino acids. This mutant, unlike the basic patch mutant, is reduced for Glc7 binding. However, from figure 1B, it looks like amino acid 80 is either the "F" of the RVSF motif or the residue immediately following. Thus, it's not clear if the RVSF mutant is functional in this Spc105 fragment.*"

Author's response: The phenylalanine residue is downstream from the 'RVSF' motif. However, reviewer 2 also pointed out that this mutation may impact the RVSF motif activity. Our intention in this experiment was to confirm that reduced PP1 recruitment improves chromosome biorientation. The *in vitro* pull-down confirms reduced PP1 recruitment, while the benomyl assay confirms improved reduced chromosome mis-segregation. Nevertheless, with the direct evidence in the form of kinetochore particle pull-down in hand, we decided to remove this data.

- *"(2) The authors find that Spc105-BPM expressing cells exhibit enhanced recruitment of Bub3 to kinetochores (Figure 1F). The two hypotheses they present to explain this are: (1) mutating the basic patch results in decreased Glc7, which leads to decreased dephosphorylation (increased/retained phosphorylation) of the MELT motifs thus and high Bub3 recruitment; (2) mutating the basic patch prevents Spc105 from binding MTs, and this prevents a conformational change that would normally occur upon MT binding that results in structural reorganization of the kinetochore, preventing Mps1 from phosphorylating the MELT motifs. To distinguish between the two, they generate constructs in which they add back a basic patch alone with "surrounding residues" (to presumably rescue MT binding) or a basic patch plus the RVSF motif (canonical Glc7 recruitment domain). They find that the basic patch alone does not suppress Bub3 recruitment but the basic patch plus RVSF does. They then conclude that the MT*

binding activity of the basic patch cannot be responsible for the observed results. Two things here: (1) Did the authors test that adding back the basic patch indeed rescued MT binding? If not, the experiment is inconclusive. (2) The authors conclude that the data "strongly suggest that the basic patch and the RVSF motif act together to recruit Glc7 activity in the yeast kinetochore..." Because the basic patch did not suppress Bub3 retention but RVSF plus the basic patch did, this result indicates that RVSF is important for recruiting Glc7, but doesn't address the role of the basic patch (except that it is not sufficient to recruit Glc7). It would be useful for the authors to add back RVSF (plus surrounding residues) alone and compare the retention of Bub3 to that of adding back RVSF plus the basic patch. If the basic patch has a role in Glc7 recruitment, one would predict a differential response to Bub3 retention."

Author's response: These are both good suggestions. We decided against testing the microtubule-binding ability of the extra basic patch, because *in vivo* data shows that microtubule-binding is not important for kinetochore force generation. We did, however, conduct two additional experiments to further support the idea that the basic patch assists the RVSF motif in recruiting Glc7.

First, as suggested by this reviewer, we add back the RVSF motif to $Spc105^{BPM}$ either with ($Spc105^{55\text{-}145}$) or without the basic patch ($Spc105^{55\text{-}145,\ 101\text{-}104::AAAA}$). Consistent with our expectation, Bub1 or Bub3 recruitment is suppressed only when the RVSF motif is introduced with its own basic patch.

These data are shown in Fig. 1F i and ii. Also please check the genetic interaction between these fusion chimeras and *bub1*$^{\Delta kinase}$ in Fig. S2D.

Second, we also fused a known plus-end binding domain, TOG2 from the protein Stu2/XMAP215 to $Spc105^{BPM}$. However, this addition did not suppress Bub3 recruitment. These data are shown in Fig. 1F iii.

These data are shown in Fig. 1F iii.

- *"(3) Throughout the paper, the authors propose and conclude that the basic patch is important for recruiting Glc7 to kinetochores. In the first three figures of the paper, the authors utilize the basic patch mutant to probe how decreased levels of Glc7 affect various aspects of kinetochore function. However, based on the above two points, the authors cannot conclude that the basic patch has a clear role in Glc7 recruitment. Because of this and the fact that the basic patch has an additional function in microtubule binding, the binding patch mutant is not appropriate as a tool to test biological outcomes of reduced Glc7 recruitment."*

Author's response: We show that the kinetochore particle pull-down experiments demonstrate that the basic patch aids the RVSF motif in promoting Glc7 recruitment.

- *"(4) Another major conclusion the authors draw is that because the binding patch mutant reduces Glc7 recruitment to kinetochores, this results in "faster kinetochore bi-orientation." It is not clear that this is the case from the data presented in Figure 1D (and for Figure 4B). They show SPB-kinetochore and kinetochore-kinetochore separation at two time-points after release from a G1 arrest. A couple of things here: (1) There is no rate information shown. Is the conclusion that they bi-orient faster drawn from the fact*

that the two kinetochore masses are farther apart at the two time points? (2) It is also not clear if the kinetochores are properly bi-oriented. The Danuser lab showed that the separation of the two kinetochore clusters is not a reliable read-out for bi-orientation - rather the CENs need to be labeled and tracked (Marco et al, 2013). Given that the conclusion reached from this assay (kinetochores bi-oriented faster and correctly) becomes an assumption for almost all subsequent experiments, the authors should confirm their results using a more high-resolution assay."

Author's response: We have now added centromere tracking (using a centromere proximal TetO array) to show that a larger fraction of cells achieves chromosome IV biorientation in basic patch mutant cells as compared to wild-type cells.

These data are shown in Fig. 1C.

- *"A separate point here is that a predominant phenotype in Figure 1D (and 4B) appears to be the increased spread/scatter of the kinetochore clusters, which may be indicative of faulty bi-orientation or some other defect. This is an important point - since, as mentioned above, the authors use the conclusion that the basic patch mutant results in "better" error correction as assumptions for the remaining experiments in the paper."*

Author's response: The appearance of an increased scatter is entirely due to data processing. As discussed in the methods, to construct the V-plot, we first project the 2-D intensity distribution on the spindle axis (by computing a column-wise sum of intensities perpendicular to the spindle axis). Next, we normalize this value by dividing it with the total sum of all pixels. If the kinetochores are separated by a larger distance i.e. over more pixels, then the intensity of each pixel becomes lower. In any case, the centromere-labeling experiment confirms that the kinetics of kinetochore biorientation and distance between sister centromeres increases in basic patch mutants. This is discussed in the methods section of the manuscript in page 20.

- *"And finally in Figure 1D, the authors measure an increase in kinetochore-kinetochore distance. They conclude that expression of the binding patch mutant results in "enhanced force generation" (stronger kinetochore - microtubule attachments). This cannot necessarily be concluded from the k-k distance data alone. Just as an example, wouldn't a similar phenotype be observed in the case of defects in the cohesin pathway or in mis-regulation of chromatin organization? In addition, the conclusion that kinetochore-microtubule attachments are stronger (generate more force) is not consistent with their model that recruitment of PP1 results in stable kinetochore-MT attachments while reduced recruitment promotes kinetochore-MT turnover ('error correction'). How do the authors explain how a decrease in PP1 might increase kinetochore-MT forces?*"

Author's response: This concern echoes a comment made by reviewer 2 (page 8-9 of this document). There is a very simple explanation for the observation that decrease in PP1 activity, *specifically by weakening the Spc105-PP1 interaction,* leads to increased kinetochore forces.

The accepted notion in the field proposes that PP1 recruited by Spc105 stabilizes kinetochore-microtubule interactions. However, there is actually very little data showing that PP1 specifically recruited by Spc105 stabilizes kinetochore-microtubule attachments. In fact, results from Rosenberg et al (Current Biology 2011), which are now confirmed by us, show that *PP1 recruited by Spc105 is not required for stabilizing kinetochore microtubule attachments*.

The positive genetic interactions between Spc105^{BPM} and Spc105^{RASA} with nearly every major factor involved in kinetochore biorientation and error correction along show that PP1 recruited by Spc105 is in fact harmful. If Spc105-Glc7 interaction is weakened, a stringent error correction mechanism is no longer necessary (assays involving *ipl1-2*, *ndc80-6A dam1-3A*). We also directly demonstrate this by artificially tethering Glc7 to Spc105. Also note the suppression of benomyl lethality of *rts1Δ* cells. In this case, centromeric enrichment of Sgo1 is known to be unaffected.

In conclusion, weakening Glc7-Spc105 interaction results in a lower incidence of stable syntelic attachments and/or faster kinetochore biorientation, and this improves chromosome segregation accuracy.

We clearly state this model on page 13-14.

- *"(5) In figure 2C, the authors report that cells expressing the binding patch mutant have a slight delay in transitioning from metaphase to anaphase. They suggest that the delay is a result of reduced Glc7 recruitment and therefore increased pMELTs and SAC protein retention. They use this as further evidence that the binding patch mutant recruits Glc7. Two questions: (1) is this difference significant? (2) are there other explanations for the slight delay (e.g. defects in chromosome biorientation as observed in Figure 1D?)"*

Author's response: As we discussed above, Figure 1D does not demonstrate defective chromosome biorientation. Since the delay is relatively minor and given the preponderance of other data, we decided not to pursue this experiment any further.

- *"(6) In Figure 3, the authors show that the Spc105 binding patch mutant rescues lethality of a Sgo1-deletion. Sgo1 is involved in the "error correction" pathway (by recruiting the CPC) and its loss would presumably result in more stable kinetochore-microtubule attachments and a decrease in error correction efficiency. Because the binding patch mutant rescues Sgo1Δ, the authors use this as further evidence that the binding patch recruits Glc7, with the rationale being that the binding patch mutant recruits less Glc7, resulting in higher kinetochore-microtubule turnover, which in turn rescues cells from the proposed hyper-stable kinetochore-microtubules in sgo1Δ cells. Given the role of the basic patch in binding microtubules, an alternative explanation is that the loss of Spc105-mediated microtubule binding in cells expressing the basic patch mutant rescues the hyper-stable attachments in the Sgo1Δ cells."*

Author's response: We would like to draw attention back to the suppression of *ndc80-6A* and *dam1-3A* mutants, which make hyper-stable kinetochore-microtubule attachments (Cheeseman et al., 2002; Lampson et al., 2004; Pinsky et al., 2006; Tanaka et al., 2002). These mutants are sensitive to benomyl-containing media, especially the double mutant *ndc80-6A dam1-3A*. This indicates that hyper-stable kinetochore-microtubule attachments do not counter-act the effects of benomyl. Furthermore, our old and new measurements of centromere and kinetochore separation in Spc105^{BPM} cells show that kinetochore-microtubule attachments are not destabilized as a result of Spc105^{BPM}. The same is also true of the suppression of *rts1Δ* by Spc105^{BPM}.

We mention the hyper stable attachments of ndc80-6A and dam1-3A mutants on Page 9.

- *"(7) In Figure 4E, what is the rationale for testing chromosome alignment/error correction in cells expressing the Spc105-RASA mutant in the Sgo1Δ cells? As an extra challenge to error correction? Is the effect still observed in wild-type cells? Also, in the graph shown in figure 4E, bottom left, are the differences between WT and mutant Spc105 significant at the two time points?*"

Author's response: Observing an improvement in chromosome segregation in budding yeast under normal conditions is quite difficult: the error probability is predicted to be 1 in 10,000 cell divisions. Therefore, almost all of the assays used here rely on challenging the cells in some manner (benomyl sensitivity, sgo1Δ, see e.g. Peplovska et al). Hence, we tested the chromosome segregation in *sgo1Δ* strains in Fig. 4A.

We are sorry that we omitted the statistical significance information in this figure. The difference between the mutant and wild-type is statistically significant. In Fig. 4A, for the bar graph showing fraction of cells with bioriented *CENIV* we performed two-way ANOVA test to obtain p=0.0066 at 30 min, p=0.0043 at 45 min. For the graph showing fraction of anaphase cells with mis-segregation we performed t-test to obtain p=0.0079. We used *sgo1Δ* background mainly as an additional challenge. We now mention this in the figure legend of Fig 4A on page 28.

- *"(8) The authors demonstrate that premature recruitment of Glc7 to the N-terminus of Spc105 in early mitosis (using an FRB-FKBP approach) results in defects in chromosome alignment/biorientation (Figure 5). This provides evidence that Ipl1 needs to phosphorylate Spc105 to prevent premature loading of Glc7 and likely premature stabilization of kinetochore-MT attachments. In the Rosenberg, 2011 study, the authors use an Spc105 RVAF mutant for the same ends - to constitutively recruit Glc7 to kinetochores. In this case, the authors reported no defects in cell cycle progression or chromosome segregation. Have the authors here tested a similar mutant? Is it possible that fusing Glc7-FKBP to FRB-fused Spc105 causes unintended problems in early mitosis? They show that these modules/fusions don't affect chromosome segregation when targeted in late mitosis after biorientation has occured, but it could be that these fusions only cause issues during the process of generating attachments. It seems the RVAF mutant might be a more straightforward way to address this, as it should also prematurely recruit Glc7 to kinetochores."*

Author's response: We obtained and studied chromosome biorientation in the Glc7-Spc105^{RASA} fusion strain constructed by Rosenberg et al. In our hands, we found that a significant fraction of cells from this strain contained unattached or misaligned kinetochores (as compared to a wild-type strain from the same strain background). Also, this strain is sensitive to the typical benomyl concentrations that we use in the lab (30 µg/ml). These effects could be due to the untimely PP1 activity in the kinetochore as we hypothesize. However, we were also concerned about the potential effects of the ever-present PP1 on kinetochore assembly.

These data are now displayed in Fig. S3. The discussion of our results with Glc7-Spc105 RASA is included on page 12.

- *"Additional comments:*
  1. The authors conclude that the basic patch / MT binding domain (in addition to the RVSF motif) in Spc105 is important for Glc7 recruitment in budding yeast. This is

somewhat different than the case in C. elegans, where the two domains have distinct and non-overlapping activities (Espeut et al., 2012) This potential difference across systems is worth mentioning. Related to this, it is probably also important to note that in C. elegans, the KNL1 MTBD is also involved in forming load-bearing kinetochore-MT attachments, suggesting that the roles of KNL1-recruited may be different across species."

Author's response: We have noted these differences in page 4, paragraph 1 and discussed in page 14, paragraph 2.

- *"2. In a couple of instances, the phrase "error correction" is used, but I think the authors mean "attachment stabilization." For example: Page 12, bottom: "We find that Glc7 activity required for error correction and SAC silencing is derived from independent sources." Glc7's "activity" is kinetochore-MT stabilization, whereas its absence (according to the authors) is required for kinetochore-MT turnover or "error correction." [same for Page 12, bottom, "The source of Glc7 activity used in error correction is unclear."]"*

Author's response: This is a good point. We have edited the manuscript significantly to indicate that reduced Glc7 recruitment via Spc105 leads to faster chromosome biorientation.

- *"3. A recent paper (Suzuki et al., 2018) reported that in budding yeast, Cin8 recruits a population of PP1/Glc7 to kinetochores specifically in late mitosis to generate tension (increase binding) between Ndc80 complexes and microtubules. This suggests a recruitment mechanism for the "other" population of PP1 that the authors here propose, but do not identify. This relevant study should be mentioned."*

Author's response: We have added this reference in the discussion on page 14.

- *"4. As rationale to explore the Spc105 basic patch as a potential recruiter of Glc7, the authors state, "Finally, basic patch activity can be disrupted by phosphorylation of the Serine and Threonine residues immediately downstream." Is this from previously published work? A citation or Figure callout would be helpful."*

Author's response: We now include the citation as follows:

Page 4: [pSYT repository of phosphorylated peptides hosted at the Global Proteome Machine Database; also see (Kanshin et al., 2017; Smolka et al., 2007)].

Page 13:http://gpmdb.thegpm.org/psyt/index.html.

We mentioned the complete webpage url in Methods section in page 18 which can be used to check the phosphorylation sites of Spc105.

- *"5. In the main text or in Figure 1, it would be useful to state how the basic patch residues RRRK were mutated to generate the basic patch mutant.*"

Author's response: We have mentioned this in the result section in page 4 and in Methods section in page 16.

- *"6. Page 4: What is meant by "single molecule microscopy?" Are we looking at single molecules in Figure 1? My interpretation from the text is that many molecules are coated on a bead.*"

Author's response: The statement refers to our single molecule observations which failed to show any interaction between single GFP-labeled phosphodomain molecules and stabilized microtubules. To clarify this, we have changed the statement as follows:

Page 4: 'We first used Total Internal Reflection Fluorescence microscopy, to test whether single molecules of a recombinant Spc105 phosphodomain (residues 2-455 as a part of 6xHIS-MBP-Spc105$^{2\text{-}455,\ 222::GFP}$) interact with Taxol-stabilized porcine microtubules but did not detect any interaction (data not shown). Therefore, we next coated microspheres with wild-type Spc105 phosphodomain molecules.'

- *"7. Page 4: What is meant by "recombinant phospho-domains?" The N-terminal region, the MELT motifs, all of them together?"*

Author's response: We fused the fragment containing 2-455 amino acid residues of Spc105 with 6xHIS-MBP and expressed the chimera protein in *E. coli* (see page 4 for brief description. See methods in page 17-18 for construction of the plasmid and page 21 for expression and purification protocols).

- *"8. Figure 1E: The authors state in the text that Mad1 is not detectable (and not changed) in WT or mutant cells. By my eye, it looks increased. It would be helpful to see a quantification of Mad2 in control, early prometaphase cells or nocodazole-treated, so that the reader has a reference of what maximally high levels would be. The same could be said for Bub3."*

Author's response: There is one key detail of this experiment that we should have clarified. For Mad1 localization, we use *nup60Δ* background. Nup60 is a non-essential nuclear pore protein that tethers Mad1 to the nuclear pore making quantitative analysis of its kinetochore localization difficult (see e.g. Aravamudhan et al. 2015, Wozniak paper). A consequence of NUP60 deletion is that one or more Mad1 puncta can be seen to form in the nucleus. Sometimes, these puncta appear to colocalize with kinetochores. In the original micrograph, this appeared to be the case. Importantly, the colocalization appeared with only one of the two kinetochore clusters indicating that this wasn't Mad1 recruitment by kinetochores. We have removed this non-representative micrograph and substituted it with a more representative one. We are happy to include a gallery if necessary.

The reviewer is right in concluding that there is a small increase in the number of metaphase-arrested cells with visible Bub3 and Mad1. We have now used the appropriate statistical analysis (Fisher's exact test for binomial fractions) to confirm whether the observed increase is statistically significant.

Regarding Mad2 localization, although Mad2-GFP and GFP-Mad2 have been used, fluorescently labeled Mad2 is not functional in yeast (and in human cells). Therefore, we rely on our previous Mad1-GFP or Mad1-mCherry localization data (Aravamudhan et al., 2015, Aravamudhan et al., 2016).

- *"9 Page 7: The authors state that "Higher Sgo1 recruitment will in turn enhance sister centromere cohesion and Aurora B activity thus resulting in better biorientation and error correction." More Aurora doesn't necessarily mean "better" biorientation and correction - sometimes means worse."*

Author's response: We have changed this statement as follows:

Page 7-8: "One potential explanation for this phenotype is that a higher Bub1 recruitment to the kinetochore will promote Shugoshin (Sgo1) recruitment (Fig. S2A), enhancing sister centromere cohesion and Aurora B activity". Both these effects will promote sister kinetochore biorientation (Kawashima et al., 2010a; Kawashima et al., 2010b; Peplowska et al., 2014; Salic et al., 2004).

August 16, 2019

Re: JCB manuscript #201810172R-A

Dr. Ajit Joglekar
University of Michigan
109 Zina Pitcher, 3067 BSRB
Ann Arbor, Michigan 48109

Dear Ajit,

Thank you for submitting your revised manuscript entitled "Minimization of a harmful cross-talk between mitotic checkpoint silencing and error correction". The manuscript has been evaluated by 3 expert reviewers (Rev#1 evaluated the earlier version, the other 2 reviewers are new as the prior reviewers were unable to re-re-review. New referees were instructed that this was a revision and were asked to assess whether the paper was now sufficiently developed for publication, consistent with our policy limiting all papers to one round of major experimental revision). The expert opinion is mixed, with Reviewer #4 being the most critical, Reviewer #1 in the middle and Reviewer #5 more supportive.

After consulting Reviewer #1 (who had evaluated the initial submission) and based on our internal discussions, we believe that a major issue lies in how the data in the manuscript is presented and interpreted. Reviewer #1 remains concerned with the lack of clarity around what exactly the benomyl-sensitivity assay tests. They discussed with us that many interesting results stem from that assay, which is difficult to interpret mechanistically. In addition, see the comments from Rev#4 regarding a number of claims that are emphasized in the text but not substantiated by the data, comments that were echoed by the other referees. It seems from our reading that the mutation in the basic patch has created a weaker PP1 binding mutant of Spc105/Knl1 than the classic RRASA mutant. Thus, much of the data could be interpreted in terms of different thresholds of PP1 activity being required for biorientation versus checkpoint silencing as opposed to different pools. Some additional analysis of the weaker GILK mutant should be conducted to assess if this is indeed the case, which would greatly simplify interpretations (since the GILK binding to PP1 is well characterized). In addition to addressing this major conceptual issue and modifying the title/abstract in response to the reviewer feedback, we also ask that you address the comments from the reviewers on the analysis of PP1 binding in the basic patch mutant as well as other comments and list them in a response to reviewers.

We would be happy to talk with you about the decision to help clarify any questions you may have.

Our general policy is that papers are considered through only one revision cycle; however, given that the suggested changes are intended to clarify and improve the manuscript, we are open to one additional short round of revision. Please note that we will expect to make a final decision without additional reviewer input upon resubmission.

Please submit the final revision within one-two months, along with a cover letter that includes a point by point response to the reviewer comments.

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

Sincerely,

Arshad Desai, PhD
Editor, Journal of Cell Biology

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

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

In the revised manuscript by Roy et al., the authors improve on their results concerning the role of PP1 recruitment by Spc105 in chromosome segregation. The authors' key finding that Spc105-recruited PP1 can rescue robust chromosome segregation independently of the two known pathways downstream of the MELT repeat phosphorylation has been substantially strengthened by the additional experiments. This conclusion is quite interesting and will move the field forward in understanding chromosome biorientation, many aspects of which are still poorly understood. With some changes and additions to the text, I recommend the article for publication.

Minor issues:
1. One of my previous issues concerned the authors' claim that "Spc105BPM improves the accuracy of chromosome segregation." This statement makes it sound like yeast strains with this mutation would missegregate their chromosomes less often than a wild-type strain. In their response, the authors claim "Observing an improvement in chromosome segregation in budding yeast under normal conditions is quite difficult because segregation errors are rare." I agree, which is why such a statement is impossible to make. None-the-less, the authors make this claim repeatedly in the revised manuscript, including:
"These results reveal that spc105BPM improves chromosome segregation." p. 9
"spc105BPM improves the accuracy of chromosome segregation. " p. 10
"Thus, Spc105BPM improves the kinetics of kinetochore biorientation and reduces chromosome segregation errors. " p. 10 (Is faster biorientation necessarily "improved"?)
"cells expressing Spc105BPM exhibit improved accuracy of chromosome segregation." p. 10

It would be much more accurate and less confusing to consistently state which mutations and pathways are being suppressed by Spc105BPM to improve chromosome segregation relative to those mutations rather than these blanket statements. For example, if you combined these mutations with phosphomimics of the Ipl1 sites on Dam1, I doubt it would "improve the accuracy of chromosome segregation" in that case.

2. The authors claim that "Spc105BPM does not enhance SAC signaling" when they see a very strong increase in Mad1 recruitment to kinetochores and an anaphase delay in cells with this mutation. The authors could claim this is a mild effect in comparison to the SAC arrest seen in the RASA mutation, but to claim that it does not enhance the signaling and that there is a "lack of Mad1 recruitment" is in direct contradiction with the data presented.

3. The new kinetochore pull-down assay has some confusing results. The amount of Spc105 that comes down with the kinetochore is wildly inconsistent, suggesting that either the quantification or the overall composition of the kinetochore particles is not reproducible. It is therefore a bit difficult to find differences in the phosphatase levels very convincing. Minimally, the authors should state how the quantification of the western blots was performed in the methods, including how the imaging was performed (direct imaging vs. film).

4. In the abstract, the authors state that "We show that to mitigate this cross-talk, the yeast kinetochore uses independent PP1 sources to stabilize correct attachments and to silence the SAC, and also delays the recruitment of PP1 for SAC silencing." I would argue that the authors don't show a delay in PP1 recruitment, as this was not tested directly. This is more a proposition or model based on their results.

Reviewer #4 (Comments to the Authors (Required)):

This manuscript addresses an interesting idea - that PP1 recruitment to kinetochores to silence the SAC may interfere with error correction (referred to as harmful cross-talk). The data show that artificial tethering of PP1 causes problems for chromosome biorientation, which justifies the idea that premature PP1 recruitment could be problematic. Beyond that point, there is a lot of data, but the argument is difficult to follow. The solution to the potentially harmful cross-talk, as written in the abstract, is that the "kinetochore uses independent PP1 sources to stabilize correct attachments and silence the SAC, and also delays the recruitment of PP1 for SAC silencing." The evidence supporting these ideas is unclear.

Here are the main findings that I see in the paper:
1. The authors identify a basic patch that can recruit PP1, in addition to the previously known RVSF and GILK motifs. The first two Results sections shows that mutation of this basic patch affects biorientation, SAC silencing (Bub3 removal from kinetochores), and PP1 recruitment (based on kinetochore particle pull-down). The next section seems to partially contradict the SAC silencing point (p. 8: "Spc105-BPM ... causes at most a minor delay in SAC silencing"), however, which is confusing.
2. The basic patch mutant improves chromosome segregation under several conditions such as benomyl and sgo1 deletion, which implies that it is involved in regulating kinetochore-microtubule interactions.
3. Mutation of the RVSF motif in Spc105 also improves chromosome segregation with benomyl, which seems similar to the basic patch mutant.
4. Artificial tethering of PP1 to Spc105 causes biorientation problems, which motivates the idea of harmful cross-talk as discussed above.
5. Mutations of phosphorylation sites near the basic patch has effects on SAC silencing and chromosome segregation similar to the basic patch mutant. It's not clear how phosphorylation of these sites is regulated normally.

Based on these findings, I don't see the evidence for "independent PP1 sources" to solve the potential problem of harmful cross-talk. What are the independent sources? Mutation of either the basic patch or RVSF seems to have similar effects. I also don't see direct evidence for delayed PP1 recruitment for SAC silencing. If the data are there, they should be more clearly explained.

Additional comment:

I found the presentation of Figure 1F very confusing. There are many different labels, and they don't seem to be used consistently. For example, the text refers to RVSF + BP + ABPM (which corresponds to one of the schematics in the figure) and RVSF + BP + BPM (which does not). In the graphs, the rationale for all the different comparisons is not clear. Also, the text says TOG2 + BP for panel 1G, and the figure says TOG2 + ABPM. With so many different Spc105 mutations and appendages, the nomenclature needs to be consistent and each one clearly explained.

Reviewer #5 (Comments to the Authors (Required)):

The authors have carried out many new experiments to address the concerns of the reviewers and their new data and re-writing have significantly improved the manuscript. With a few relevant changes, I am happy to recommend publication.

- kinetochore particles have now been purified from cells, demonstrating a significant reduction in Glc7 binding to the Spc105 basic patch mutant (62-85% decrease, Fig. 2A and S1E).
The quantitation here varies significantly, for both Spc105 and PP1. The reduction in PP1 binding is clear, but perhaps a third repeat should be performed to improve quantitation?
It would have been nice to see this binding defect rescued by an additional RVSF motif + BP, and to be compared to Glc7 binding defects in GILK and RVSF mutants, but I don't feel that this is necessary for publication.

- the GILK motif is shown to cause similar, albeit weaker, phenotypes (enhanced Bub3 recruitment etc.).

- TetO-TetR-GFP marked CENIV experiments demonstrate faster bi-orientation in the Spc105 basic-patch mutant, compared to wild-type cells (Fig 1C). Theses centromeres are also separated slightly more, at 45 mins (after release from G1 arrest).

Points to be addressed/clarified:
In the abstract: the authors state that "the yeast kinetochore uses independent PP1 sources...." to avoid problems of cross-talk between the regulation of bi-orientation and SAC signalling. It is not made clear what the two sources of PP1 are. Presumably one is the Spc105-bound pool, what is the other source?

Page 8. The sub-title used here is that the BPM mutant significantly "improves" chromosome segregation. The authors should be careful here. They demonstrate (Fig. 3) that this mutation can suppress the benomyl sensitivity of several mutants with defective segregation defects (sgo1, bub1Δkinase, ndc80-6A, dam1-3A etc). This can be explained as the mutants tested are all likely to lead to, or mimic, decreased Aurora kinase activity at kinetochores. By decreasing Glc7 levels at kinetochores, the spc105-BPM restores the 'balance' of kinase-phosphatase levels. Thus the BPM can improve chromosome segregation in mutant backgrounds.
They demonstrate faster bi-orientation than a few hundred wild-type cells (Fig. 1C), but not better/improved chromosome segregation than wild-type cells in general. To test that they could score mini-chromosome loss in tens of thousands of cells - is this something that the authors really think the BPM will do? Please clarify the text or analyse segregation efficiency in other ways.

The KNL1/Spc105 RVSF motif is known to be phosphorylated in S. cerevisiae cells, but is there any data demonstrating that this is actually carried out by Ipl1/Aurora kinase (outside of humans)? If

not, then it should not be assumed - other kinases may be responsible and this should be clarified in the text.

Page 5, line 3, should be Fig. S1C (not S1B).

Page 11 middle paragraph: A weakened interaction between Glc7 and PP1.....

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

> *"Minor issues:*
> *1. One of my previous issues concerned the authors' claim that "Spc105BPM improves the accuracy of chromosome segregation." This statement makes it sound like yeast strains with this mutation would missegregate their chromosomes less often than a wild-type strain. In their response, the authors claim "Observing an improvement in chromosome segregation in budding yeast under normal conditions is quite difficult because segregation errors are rare." I agree, which is why such a statement is impossible to make. None-the-less, the authors make this claim repeatedly in the revised manuscript, including:*
> *"These results reveal that spc105BPM improves chromosome segregation." p. 9*
> *"spc105BPM improves the accuracy of chromosome segregation. " p. 10*
> *"Thus, Spc105BPM improves the kinetics of kinetochore biorientation and reduces chromosome segregation errors. " p. 10 (Is faster biorientation necessarily "improved"?)*
> *"cells expressing Spc105BPM exhibit improved accuracy of chromosome segregation." p. 10"*
>
> *It would be much more accurate and less confusing to consistently state which mutations and pathways are being suppressed by Spc105BPM to improve chromosome segregation relative to those mutations rather than these blanket statements. For example, if you combined these mutations with phosphomimics of the Ipl1 sites on Dam1, I doubt it would "improve the accuracy of chromosome segregation" in that case."*

Author's response: We understand this criticism. Improved chromosome accuracy in cells expressing Spc105^{BPM} even compared with wild-type cells is strongly implied by our data, but we haven't explicitly tested this implication. Therefore, we have edited the manuscript at several instances, including the ones pointed out by the reviewer above, to clearly state the findings from our data.

Page 3, last three sentences and page 4 first paragraph: "We find that a patch of basic residues in the N-terminus of Spc105 contributes to PP1 recruitment. We also show that in strains with impaired chromosome biorientation, the phosphoregulation of the basic patch activity improves the accuracy of chromosome segregation. Ultimately, our results demonstrate the presence of a harmful cross-talk between SAC silencing and error correction, explain how this cross-talk is likely to be mitigated in budding yeast, and also uncover a novel role for the phosphoregulation of PP1 recruitment via Spc105."

Page 9, end of second paragraph: "Strikingly, Spc105^{BPM} suppressed these defects, indicating that the accuracy of chromosome segregation is significantly improved in these strains."

Page 11, first paragraph: "Together, these conclusions imply that a weakened Spc105-Glc7 interaction impairs SAC silencing but improves the accuracy of chromosome segregation when chromosome biorientation is challenged either by mutations in a wide range of genes involved in the process or by microtubule destabilization due to benomyl."

Page 12, last sentence of first paragraph: "Thus, the improved accuracy of chromosome segregation in these strains is not because of delayed SAC silencing."

To further bolster our manuscript, we have also added results of two key experiments. First, we compare the accuracy of chromosome segregation in wild-type and *spc105^{BPM}* strains in media

containing benomyl (Fig. S2F). We find a significantly lower incidence of chromosome missegregation in *spc105^{BPM}* cells than in wild-type cells. We also measured the growth of the two strains in media containing benomyl using a plate-reader, we found that *spc105^{BPM}* cells grow significantly faster that wild-type cells. This finding suggests that the lower incidence of chromosome missegregation allows *spc105^{BPM}* cells to grow faster even than the wild-type cells. These data are discussed as follows:

Page 10, towards the end of second paragraph: 'Following this result, we assessed whether Spc105^{BPM} also increases the accuracy of chromosome missegregation as compared to wild-type Spc105 when chromosome biorientation is challenged by benomyl treatment. When mutant and wild-type cells were synchronized in G1 and then released into the cell cycle in benomyl containing media, we found that *CEN IV* marked with TetO repeats mis-segregated more frequently in wild-type cells as compared to the *spc105^{BPM}* mutants. Moreover, the *spc105^{BPM}* cells grew significantly faster in benomyl containing as compared to wild-type cells (growth measured by monitoring OD600, Fig. S2F)'.

> *"2. The authors claim that "Spc105BPM does not enhance SAC signaling" when they see a very strong increase in Mad1 recruitment to kinetochores and an anaphase delay in cells with this mutation. The authors could claim this is a mild effect in comparison to the SAC arrest seen in the RASA mutation, but to claim that it does not enhance the signaling and that there is a "lack of Mad1 recruitment" is in direct contradiction with the data presented."*

Author's response: As shown in Fig. 1E, we see visible Mad1 recruitment to bioriented kinetochores only in ~ 20% cells expressing Spc105^{BPM}. Quantification of mad1-mCherry signal in Fig. S1D confirms this. Finally, the growth of wild-type and *spc105^{BPM}* cell cultures is virtually identical (Fig. S1F). Perhaps the micrograph in Fig. S1D was misleading. We should have mentioned that the strains used for this set of experiments lacked the nuclear pore component *NUP60*. The *nup60\Delta* mutation eliminates Mad1 binding to the nuclear pore, but creates a bright aggregate of Mad1-mCherry in the nucleus (Scott et al MBoC). Importantly, this aggregate does not colocalize with kinetochores as can be seen in the micrographs in Fig. S1D. We have now clearly indicated this by adding an asterisk to the micrograph.

Page 33, Fig. S1D legend: "Representative images of metaphase cells expressing the indicated protein. The asterisk in the merged image denotes a nuclear Mad1-mCherry aggregate that forms in the absence of the nuclear pore protein Nup60 (Scott et al., 2005)."

The reviewer is correct in pointing out that there is a minor delay in anaphase onset as shown in Fig. 2D. The original heading referred to the absence of stronger checkpoint signaling from unattached kinetochores. To highlight both these points, we have changed the heading for this section as follows:

Page 7 Heading of the last paragraph: "Spc105^{BPM} does not enhance SAC signaling from unattached kinetochores, but causes a minor delay in metaphase to anaphase transition"

Page 8, end of first paragraph: "Together, these results show that Spc105^{BPM} does not affect SAC signaling from unattached kinetochores but causes a small delay in anaphase onset.."

"3. The new kinetochore pull-down assay has some confusing results. The amount of Spc105 that comes down with the kinetochore is wildly inconsistent, suggesting that either the quantification or the overall composition of the kinetochore particles is not reproducible. It is therefore a bit difficult to find differences in the phosphatase levels very convincing. Minimally, the authors should state how the quantification of the western blots was performed in the methods, including how the imaging was performed (direct imaging vs. film)."

Author's response: In this assay, the amount of Spc105-mCherry co-precipitating with Dsn1-Flag varies for each lysate, even after careful equalization of protein concentrations in the clarified lysates. Therefore, we decided to display the results of western blotting of samples from one experiment (Fig. 2A) directly. In the second experiment, we adjusted the volume of the sample loaded to account for the difference in the amount of co-precipitated Spc105-mCherry (Fig. S1E). This information is now included in the Methods section. Additionally, details of the method of imaging of the western blots is also included as follows:

Page 7, 2nd paragraph: "Briefly, we immunoprecipitated Dsn1-Flag to pull down kinetochore particles from yeast cells expressing either wild-type $Spc105^{222::mCherry}$ or $Spc105^{BPM,222::mCherry}$, and quantified the amount of Spc105 and Glc7-3xGFP co-precipitating with the kinetochore particles (see Methods). Even though we equalized total protein concentration in each lysate prior to immunoprecipitation, the amount of $Spc105^{222::mCherry}$ co-precipitating with Dsn1-Flag varied (see Methods for details regarding the imaging and quantification used). Therefore, we normalized the amount of Glc7-3xGFP for the level of $Spc105^{222::mCherry}$ in each co-precipitate."

Page 20, 1st paragraph: "The primary antibodies were detected using Horseradish Peroxidase (HRP) conjugated secondary antibodies as per manufacturer's instructions. The resulting bioluminescence was detected and quantified using the C600 imager from Azure Biosystems. The band intensities from the western blot were measured using ImageJ."

"4. In the abstract, the authors state that "We show that to mitigate this cross-talk, the yeast kinetochore uses independent PP1 sources to stabilize correct attachments and to silence the SAC, and also delays the recruitment of PP1 for SAC silencing." I would argue that the authors don't show a delay in PP1 recruitment, as this was not tested directly. This is more a proposition or model based on their results."

Author's response: We have edited the abstract as follows:

Page 2: "We find that this dual PP1 role can be problematic: if PP1 is recruited to the kinetochore for SAC silencing prior to chromosome biorientation, it interferes with error correction. In fact, PP1 recruited to the kinetochore for SAC silencing is not necessary for attachment stabilization. We propose that the yeast kinetochore delays PP1 recruitment for SAC silencing until bipolar attachments form to ensure that chromosome biorientation precedes SAC silencing, resulting in accurate chromosome segregation."

Reviewer #4 (Comments to the Authors (Required)):

> *"This manuscript addresses an interesting idea - that PP1 recruitment to kinetochores to silence the SAC may interfere with error correction (referred to as harmful cross-talk). The data show that artificial tethering of PP1 causes problems for chromosome biorientation, which justifies the idea that premature PP1 recruitment could be problematic. Beyond that point, there is a lot of data, but the argument is difficult to follow. The solution to the potentially harmful cross-talk, as written in the abstract, is that the "kinetochore uses independent PP1 sources to stabilize correct attachments and silence the SAC, and also delays the recruitment of PP1 for SAC silencing." The evidence supporting these ideas is unclear."*

Author's response: We agree with the reviewer that we only show that the PP1 recruited through Spc105 is not required for chromosome biorientation; we did not identify the other 'independent source'. We have edited the abstract and the relevant text in the manuscript to make this clear in page 2. The same is also true regarding the delayed recruitment of PP1 for SAC silencing. We clarify this in the abstract as follows:

Page 2, paragraph 1: 'We find that this dual PP1 role can be problematic: if PP1 is recruited to the kinetochore for SAC silencing prior to chromosome biorientation, it interferes with error correction. In fact, PP1 recruited to the kinetochore for SAC silencing is not necessary for attachment stabilization. We propose that the yeast kinetochore delays PP1 recruitment for SAC silencing until bipolar attachments form to ensure that chromosome biorientation precedes SAC silencing to achieve accurate chromosome segregation'.

> *"Here are the main findings that I see in the paper:*
> *1. The authors identify a basic patch that can recruit PP1, in addition to the previously known RVSF and GILK motifs. The first two Results sections shows that mutation of this basic patch affects biorientation, SAC silencing (Bub3 removal from kinetochores), and PP1 recruitment (based on kinetochore particle pull-down). The next section seems to partially contradict the SAC silencing point (p. 8: "Spc105-BPM ... causes at most a minor delay in SAC silencing"), however, which is confusing."*

Author's response: We agree that there is a strong SAC silencing defect (i.e. abnormal Bub3 recruitment in most cells), even though this doesn't translate in a long delay in anaphase onset. We clarify these points as follows:

Page 8, end of 1st paragraph: "Together, these results show that Spc105^{BPM} does not affect SAC signaling from unattached kinetochores but causes a small delay in metaphase to anaphase transition."

> *"2. The basic patch mutant improves chromosome segregation under several conditions such as benomyl and sgo1 deletion, which implies that it is involved in regulating kinetochore-microtubule interactions.*
> *3. Mutation of the RVSF motif in Spc105 also improves chromosome segregation with benomyl, which seems similar to the basic patch mutant."*

4. Artificial tethering of PP1 to Spc105 causes biorientation problems, which motivates the idea of harmful cross-talk as discussed above.
5. Mutations of phosphorylation sites near the basic patch has effects on SAC silencing and chromosome segregation similar to the basic patch mutant. It's not clear how phosphorylation of these sites is regulated normally."

Author's response: It is technically challenging to study the temporal dynamics of the phosphoregulation of the basic patch mainly because it is likely that any of the major mitotic kinases including Cdk1, Mps1, and Ipl1 could be responsible for the regulation. Given the numerous critical functions that these kinases play, it will be difficult to isolate their roles in regulating the activity of the basic patch. Therefore, we have left this aspect unaddressed in this manuscript.

"Based on these findings, I don't see the evidence for "independent PP1 sources" to solve the potential problem of harmful cross-talk. What are the independent sources?"

Author's response: As discussed above, we do not identify the source of PP1 that is essential for attachment stabilization. We have suitably modified the abstract in page 2 to clarify this point. We also discuss the potential sources in the discussion section at the end of the manuscript.

Page 15, first paragraph: 'Efficient kinetochore biorientation in *spc105*^{RASA} cells also shows that the Glc7 activity required for attachment stabilization is derived from a Spc105-independent source. This Glc7 activity may come from diffusive interactions between Glc7 and the kinetochore. A recent study suggests that the motor protein Cin8 transports Glc7 to the kinetochore (Suzuki et al., 2018). However, it is unclear how this mechanism can deliver Glc7 preferentially to kinetochores with correct, but not incorrect, attachments. A dedicated Glc7 recruitment mechanism may not even be necessary. The inter-centromeric tension generated by sister kinetochores will also selectively stabilize bipolar attachments (Akiyoshi et al., 2010; Franck et al., 2007)'.

"Mutation of either the basic patch or RVSF seems to have similar effects.

I also don't see direct evidence for delayed PP1 recruitment for SAC silencing. If the data are there, they should be more clearly explained."

Author's response: We agree that we don't have direct evidence for this point. We have edited the abstract to stress this point:

Page2, end of the paragraph: 'We propose that the yeast kinetochore delays PP1 recruitment for SAC silencing until bipolar attachments form to ensure that chromosome biorientation precedes SAC silencing to achieve accurate chromosome segregation.'

"Additional comment:
I found the presentation of Figure 1F very confusing. There are many different labels, and they

don't seem to be used consistently. For example, the text refers to RVSF + BP + ABPM (which corresponds to one of the schematics in the figure) and RVSF + BP + BPM (which does not). In the graphs, the rationale for all the different comparisons is not clear. Also, the text says TOG2 + BP for panel 1G, and the figure says TOG2 + ABPM. With so many different Spc105 mutations and appendages, the nomenclature needs to be consistent and each one clearly explained."

Author's response: We have now greatly simplified the schematic and the nomenclature in Fig. 1F to avoid this confusion. We have also edited the manuscript in page 6 and 7 to ensure that the nomenclature is entirely consistent. Thank you for pointing this out.

Reviewer #5 (Comments to the Authors (Required)):

"The authors have carried out many new experiments to address the concerns of the reviewers and their new data and re-writing have significantly improved the manuscript. With a few relevant changes, I am happy to recommend publication.

- kinetochore particles have now been purified from cells, demonstrating a significant reduction in Glc7 binding to the Spc105 basic patch mutant (62-85% decrease, Fig. 2A and S1E). The quantitation here varies significantly, for both Spc105 and PP1. The reduction in PP1 binding is clear, but perhaps a third repeat should be performed to improve quantitation? It would have been nice to see this binding defect rescued by an additional RVSF motif + BP, and to be compared to Glc7 binding defects in GILK and RVSF mutants, but I don't feel that this is necessary for publication.

- the GILK motif is shown to cause similar, albeit weaker, phenotypes (enhanced Bub3 recruitment etc.).
- TetO-TetR-GFP marked CENIV experiments demonstrate faster bi-orientation in the Spc105 basic-patch mutant, compared to wild-type cells (Fig 1C). Theses centromeres are also separated slightly more, at 45 mins (after release from G1 arrest)."

Points to be addressed/clarified:

In the abstract: the authors state that "the yeast kinetochore uses independent PP1 sources...." to avoid problems of cross-talk between the regulation of bi-orientation and SAC signalling. It is not made clear what the two sources of PP1 are. Presumably one is the Spc105-bound pool, what is the other source?

Author's response: As explained earlier in this rebuttal, we have edited the abstract and the manuscript to avoid over-stating conclusions.

"Page 8. The sub-title used here is that the BPM mutant significantly "improves" chromosome segregation. The authors should be careful here. They demonstrate (Fig. 3) that this mutation can suppress the benomyl sensitivity of several mutants with defective segregation defects (sgo1, bub1Δkinase, ndc80-6A, dam1-3A etc). This can be explained as the mutants tested are all likely to lead to, or mimic, decreased Aurora kinase activity at kinetochores. By decreasing Glc7 levels at kinetochores, the spc105-BPM restores the 'balance' of kinase-phosphatase levels. Thus the BPM can improve chromosome segregation in mutant backgrounds."

They demonstrate faster bi-orientation than a few hundred wild-type cells (Fig. 1C), but not better/improved chromosome segregation than wild-type cells in general. To test that they could score mini-chromosome loss in tens of thousands of cells - is this something that the authors really think the BPM will do? Please clarify the text or analyse segregation efficiency in other ways."

Author's response: This criticism is on point and echoes the first point of Reviewer #1. We added supporting evidence in the form of chromosome missegregation in wild-type and spc105^{BPM} cells in media containing benomyl. Nevertheless, since we haven't measured chromosome missegregation rates under normal conditions, we have edited the manuscript in the appropriate locations to ensure that the conclusions strictly adhere to the data (result section, page 5 and page 10).

> *"The KNL1/Spc105 RVSF motif is known to be phosphorylated in S. cerevisiae cells, but is there any data demonstrating that this is actually carried out by Ipl1/Aurora kinase (outside of humans)? If not, then it should not be assumed - other kinases may be responsible and this should be clarified in the text."*

Author's response: We have revised figure 5E, left panel to indicate 'mitotic kinases' instead of Aurora B in the budding yeast schematic. We have also revised the manuscript as follows.

Page 14, first sentence of second paragraph: 'As discussed previously, the Serine and Threonine residues immediately downstream from the basic patch are phosphorylated by mitotic kinases.

Page 14, last sentence of second paragraph: 'These data show that the phosphorylation of residues downstream from the basic patch by mitotic kinases can reduce Glc7 recruitment to the kinetochore and allow effective kinetochore biorientation'.

Page 15, first paragraph: '…. the phosphoregulation of Spc105 by mitotic kinases can achieve the required temporal regulation of the Glc7-Spc105 interaction'.

"Page 5, line 3, should be Fig. S1C (not S1B).

Page 11 middle paragraph: A weakened interaction between Glc7 and PP1...."

Author's response: We have fixed these errors. Thank you for catching them!

September 12, 2019

RE: JCB Manuscript #201810172RR

Dr. Ajit Joglekar
University of Michigan
109 Zina Pitcher, 3067 BSRB
Ann Arbor, Michigan 48109

Dear Ajit,

Thank you for submitting your revised manuscript entitled "Minimization of a harmful crosstalk between mitotic checkpoint silencing and error correction". We have carefully gone over the revised manuscript and, while it has improved, we believe there remain significant issues with the writing and interpretations that will be confusing and limit the appeal to the journal's audience. To avoid back-and-forth, we have provided detailed feedback on the manuscript - see the attached file. Please note that we remain concerned that the title and abstract are not entirely appropriate - they do not report on the content of the manuscript and read as discussion points. We have significantly edited the abstract and recommend that you consider modifications of the title that emphasize the data presented (see below). We would be happy to publish your paper in JCB pending these changes and final revisions necessary 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.

1) Titles, eTOC: Please consider the following revision suggestions aimed at increasing the accessibility of the work for a broad audience and non-experts.

Title: Contribution of PP1 docked on its conserved kinetochore receptor Spc105/Knl1 to SAC silencing and kinetochore-microtubule attachment regulation

Delineating the contribution of Spc105/Knl1-bound PP1 to checkpoint silencing and kinetochore-microtubule attachment regulation

eTOC summary: A 40-word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person.
- please include an eTOC summary on the title page of your revised ms **starting 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 1D, 2D, 4D, 5AB (magnifications), S1C, S3C

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

legends.
Please indicate n/sample size/how many experiments the data are representative of: 4B, 5C

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

5) A summary paragraph of all supplemental material should appear at the end of the Materials and methods section.
- Please include ~1 brief descriptive sentence per supplemental item.

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7) Author contributions: A separate author contribution section is required following the Acknowledgments in all research manuscripts. All authors should be mentioned and designated by their full names. We encourage use of the CRediT nomenclature.

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Sincerely,

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Senior Scientific Editor, Journal of Cell Biology
