The Rabl chromosome configuration masks a kinetochore reassembly mechanism in yeast mitosis

Alberto Jiménez-Martín, Alberto Pineda-Santaella, Jesús Pinto-Cruz, Daniel León-Periñán, Sabas García-Sánchez, David Delgado-Gestoso, Laura Marín-Toral, and Alfonso Fernández-Álvarez

Corresponding author(s): Alfonso Fernández-Álvarez, Pablo de Olavide University

Review Timeline:

Submission Date:	2020-09-20
Editorial Decision:	2020-10-09
Revision Received:	2021-09-01
Editorial Decision:	2021-09-24
Revision Received:	2022-02-25
Accepted:	2022-02-25

Editor-in-Chief: Matthew Welch

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

RE: Manuscript #E20-09-0600
TITLE: "Metazoan-like kinetochore arrangement masked by the interphase RabI configuration"

Dear Dr. Fernández-Álvarez,

Dear Dr. Dr. Alfonso Fernández-Álvarez,
We have now received two reviews from experts in the field. You will see that they differ in their assessment. I have read the paper as well as the reviews and will provide a summary. As reviewer #1 points out the main novel finding in the paper is the loss of Nuf2 and ndc80 in interphase in sad1/csi1 double mutants (Fig. 4 and S1). Since the centromeres are dissociated in these cells (Fig. 3), it would be important know that the inability to see Ndc80 or Nuf2 is not just due to the decrease in intensity. What is the copy number of Cnp20 vs. Ndc80? Are you losing signal because it's 1/3 as bright? In the current manuscript centromere signal is all or none (Fig. 4). What are the limits of detection, which would tell the reader whether there is no Ndc80 or it is below some observable level.
Since Ndc80 and Nuf2 are in the same complex, the conclusions of "outer" kinetochore are based on a single complex. I agree with Reviewer #1 that it is important to examine additional proteins, Mis12 or KNL1. There was some confusion about the inclusion of Mis6 and Nuf2 in Figure 4, I agree with the reviewer I couldn't tell if they were quantified as well.
The reviewer points out the intensity measurements were made from maximum projections. I agree the sum should be used.
Reviewer 2 also suggested that it would be important to have the outer and inner labels in the same strain. This is a very important suggestion.
Considering the depth of the revisions, I am unable to suggest that the manuscript be recommended for publication. I hope that you find the reviewers comments useful for future studies in this area.

Thank you for the opportunity to examine this work. We hope that as your studies progress you will consider submitting future manuscripts to Molecular Biology of the Cell (MBoC).

If you have any questions regarding the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

Sincerely,
Kerry Bloom
Monitoring Editor
Molecular Biology of the Cell

Reviewer #1 (Remarks to the Author):

The kinetochore is a complex protein assembly on the centromeric region of chromosomes, and serves as the main attachment point for microtubules of the mitotic spindle. Proper kinetochore-microtubule contacts are important for proper chromosome segregation. Proteins within the kinetochore are broadly categorized into "inner" kinetochore proteins, closer to the centromere, and "outer" kinetochore proteins closer to or in direct contact to microtubules.

Whereas in human cells, the outer kinetochore is disassembled during interphase and reassembled for mitosis, most kinetochore proteins persist on chromosomes during interphase in both budding yeast and fission yeast.

The authors of this paper report that fission yeast cells with the double genetic modification of sad1.2 and csi1 deletion largely lose kinetochore-localization of Ndc80 and Nuf2, two outer kinetochore proteins, during interphase. The authors infer that fission yeast possesses a metazoan-like assembly/disassembly pathway for the outer kinetochore, but that this is normally hidden by the persistent association of kinetochores with the region of the nuclear envelope adjacent to the spindle pole body.

This is an interesting observation, but I feel that the support for this idea is still thin, and that the data reported in this manuscript do not represent the advance of a typical paper in MBoC.

Figure 1 quantifies signals of select kinetochore proteins over the cell cycle, but does not provide new information over what was known (e.g. Saitoh et al., 1997, Nabetani et al., 2001, Hayashi et al., 2004, Asakawa et al., 2005, Tanaka et al., 2009). That the clustering of kinetochores with the SPB in interphase is independent of microtubules (Figure 2A/B) was known as well (e.g. Ding et al., MBoC 1997, Appelgren et al., 2003). The independence of filamentous actin was new to me, but is not relevant for the rest of the paper. Figure 3 describes the search for a mutant combination that leads to prominent dissociation of kinetochores from the SPB region in interphase without impairing viability too much, and the authors successfully identify a useful combination (sad1.2 csi1D). In Figure 4, the authors then show that the inner kinetochore protein Cnp20 stays localized on kinetochores in interphase in this mutant, but Ndc80 and Nuf2 (Fig. S1) do not. To me, it is only this last piece of information that provides some

new insight - and the authors only check three out of many kinetochore proteins.

I have some obvious and easy to address questions that - when answered - would make this paper more informative:
For one, I think the idea that what is seen here is 'metazoan-type' assembly/disassembly needs strengthening. For example, do the Mis12 complex and Spc7 (KNL1 ortholog) dissociate from kinetochores as well? Is there any evidence that the regulation could be similar? This could include a sequence analysis for conserved regions, or motifs, or phosphorylation sites.

To better understand the mechanism, I would like to know about the situation where some kinetochores dissociate and some stay associated. Is the outer kinetochore only lost on the dissociating kinetochores? Is the signal loss likely to be cause or consequence of dissociation or unrelated to dissociation?
In Figure 4E, the authors do not find any interphase Ndc80 loss in single sad1.2 or csi1D mutants, although about half of these cells do show partial kinetochore dissociation from the SPB region. Does this mean that there is no correlation between dissociation and loss of outer kinetochore proteins? What about those sad1.2 csi1D cells where partial kinetochore dissociation occurs? Do all kinetochores lose signal? Or only those that dissociate? Does Ndc80 disappear before or after dissociation? What about re-tethering experiments, such as those performed in Fernández-Álvarez et al. 2016? Does that rescue Ndc80 signals in interphase in sad1.2 csi1D cells?
It seems such question would be easily addressable by the live-cell imaging approach that the authors have established.

Obviously, those are a lot of questions, but I feel that at least some of them need to be explored to add some substance to this interesting observation.

In addition, to be easily understandable (and publishable), the text of this manuscript needs intensive editing. The phrasing at times is so confusing that it becomes hard to understand the intended meaning, and some statements - in their current form - are scientifically incorrect or at least misleading.
Citations should also be carefully revised to pick the most appropriate ones. Just as one example, around line 117-120, a sentence about the situation in fission yeast is followed by two budding yeast citations.

Minor comments:
- In the title it seems like "Rabl" is mistakenly written as "RabI" or "Rab1".
- "arrangement" does not seem the most appropriate word in the title for what the authors want to report.
- Figure 4E/F legend mentions Mis6 and Nuf2, although the quantification is purely based on Cnp20 and Ndc80 as far as I can tell.
- A maximum projection was used for quantification of fluorescence signals. This will not accurately analyze the total signal. It is appropriate for analyzing distances, but not for analyzing intensities. (In my opinion, an average or sum projection should be used).
- Statistics: Figure 3G-I - At least three independent experiments were performed. It would be more informative to show variability between experiments on these graphs rather than just pooling all data.
- In Figure 3B, it was unclear to me how the arbitrary units for viability were derived, and I was surprised that lem2D csi1D cells scored so low (and show poor growth in Figure 3C), although colony size in the assay in Figure 3A seems normal.

Reviewer #2 (Remarks to the Author):

Unlike the inner kinetochore, the metazoan outer kinetochore complex is assembled before mitotic onset and then disassembled after mitosis. By contrast, yeasts constitutively display an outer kinetochore associated to the SPB (centrosome-like structure in fungi). In this ms., Jiménez-Martín and colleagues uncover a metazoan-like process of outer kinetochore recruitment during mitotic onset in fission yeast that is normally masked by the presence of the Rabl configuration. Briefly, the authors designed a genetic system that transiently abrogates Rabl configuration in interphase. This condition triggers outer kinetochore disassembly allowing the detection of outer kinetochore recruitment during mitotic onset. Overall, these observations are unexpected and therefore of outstanding interest for the kinetochore community, not only for fission yeast researchers. I really enjoyed reading this paper and I think that the ms. can be improved if the authors address the following comments, which are mostly suggestions and clarifications.

Major comments and suggestions:

_ It is essential to show data from strains in which outer kinetochore, inner kinetochore and SPB proteins are all tagged. That would convincingly show that the outer kinetochore disappears when the three centromeres detach from SPB. With the data provided, this can only be assumed.

_Is the data shown in Figs. 3, 4 and S1 obtained from expts. performed at 32C? It looks like live-cell imaging experiments were performed at 27C according to the Methods. If so, I assume that the double mutant sad1.2 csi1Δ is a hypomorph. A thorough characterization of this mutant would clarify this issue, e.g. dilution assays and liquid growth curves at different temperatures.

_ How about Mis12, KNL-1 or other outer kinetochore proteins that are not in the Ndc80 complex? I assume that such proteins

would also disappear when the Ndc80 signal is gone as occurs during meiotic prophase I (Asakawa et al., 2005; Hiraoka group).

_ Related to above: Hayashi et al. 2006 (Hiraoka group) also showed that outer kinetochore proteins are delocalized but not degraded during meiotic prophase I. I wonder if this also the case for sad1.2 csi1Δ. Western blots for outer kinetochore proteins in interphase would be feasible to perform.

_It would be nice to show the recruitment of the outer kinetochore in sad1.2 csi1Δ during mitosis with another approach (preferentially with a non live-cell imaging technique such as ChIP). Perhaps using cut9 or nda3-KM311 mutants may be a way do so.

_ Strain list must be provided.

_Supp. Figure 1: The GFP dot intensities in sad1.2 csi1Δ sees to be consistently higher than those in wt cells. I wonder if this is something related to photobleaching, or if not, this may be a meaningful observation. In any case, please provide an explanation in the figure legend or text.

Minor issues:

Title: Contains "Rabi" instead of "Rabl"

Introduction: Please cite Mizuguchi et al. papers (PMID: 25307058 and 26096785) when describing the Rabl configuration in fission yeast.

Fig. 1: For some proteins, I notice a slight decrease of the GFP signal as the cells enter mitosis. Can this observation be explained by photobleaching?

2F: Please acknowledge the reference in the figure legend (Fernandez-Alvarez and Cooper 2017b). That would help the reader.

Fig. 3A. I would call it a "strong negative genetic interaction" rather than "synthetic lethality" as some cells are still viable. In fact, the authors state "sporadically sad1.2 lem2Δ csi1Δ....". Could they add more quantification or show more tetrads?

Fig. 3B: It is not obvious what the assay actually measures unless one reads the Materials section. In any case, OD600 liquid growth curves would be more informative.

Fig. 4. E-F. Line 505 "Quantification of the centromere signals" may be better replaced with "Quantification of cells displaying centromeric signals".

Dear Editor,

We hereby submit a revised version of the manuscript E20-09-0600, entitled '*A metazoan-like kinetochore reassembly mechanism is conserved in yeast mitosis but masked by the Rabl configuration*'. We are grateful for the comments made by the reviewers and thank them for their constructive suggestions. We have addressed all the reviewers' comments and in doing so, have clarified and reinforced the new observations we are presenting. We would be very grateful if we had a new opportunity to know the opinion of the reviewers on this new version of our work.

Here, we respond to each of the reviewers' comments in turn:

Reviewer #1 (Remarks to the Author):

The kinetochore is a complex protein assembly on the centromeric region of chromosomes, and serves as the main attachment point for microtubules of the mitotic spindle. Proper kinetochore-microtubule contacts are important for proper chromosome segregation. Proteins within the kinetochore are broadly categorized into "inner" kinetochore proteins, closer to the centromere, and "outer" kinetochore proteins closer to or in direct contact to microtubules.

Whereas in human cells, the outer kinetochore is disassembled during interphase and reassembled for mitosis, most kinetochore proteins persist on chromosomes during interphase in both budding yeast and fission yeast.

The authors of this paper report that fission yeast cells with the double genetic modification of sad1.2 and csi1 deletion largely lose kinetochore-localization of Ndc80 and Nuf2, two outer kinetochore proteins, during interphase. The authors infer that fission yeast possesses a metazoan-like assembly/disassembly pathway for the outer kinetochore, but that this is normally hidden by the persistent association of kinetochores with the region of the nuclear envelope adjacent to the spindle pole body.

This is an interesting observation, but I feel that the support for this idea is still thin, and that the data reported in this manuscript do not represent the advance of a typical paper in MBoC.

We appreciate the reviewer's opinion on the relevance of our observations. We have performed new experiments with three more kinetochore proteins (Mis12, Spc7 and Dad2), new controls and improved the text of the manuscript to clarify the meaning of the data following the reviewer's suggestions. We hope that the reviewer consider this new version of the paper suitable for publication in *MBoC*.

Figure 1 quantifies signals of select kinetochore proteins over the cell cycle, but does not provide new information over what was known (e.g. Saitoh et al., 1997, Nabetani et al., 2001, Hayashi et al., 2004, Asakawa et al., 2005, Tanaka et al., 2009).

Our goal with the analysis of several kinetochore proteins during the cell cycle in *wt* cells was to rule out small fluctuations in the kinetochore signals during interphase. We agree with the

reviewer that this information is more a control of our methodology than a new result. For this reason, we have removed the old Figure 1 and distributed all these quantifications as controls along with those performed for Rabl configuration-deficient cells (Figure 2A, Figure 2D, Figure 3A, Figure 3D and Supplementary Figure 2B).

That the clustering of kinetochores with the SPB in interphase is independent of microtubules (Figure 2A/B) was known as well (e.g. Ding et al., MBoC 1997, Appelgren et al., 2003).

Thanks for the comment. In our opinion, our experiments with MBC and the quantification of the distance between centromeres and SPB are new and complementary to the two excellent previous works based on Electron Microscopy studies cited by the reviewer. We have cited both articles in the text, but we think that our approach with MBC is relevant to rule out small defects in centromere-SPB dissociation. We agree with the reviewer that, given the results, the data are not essetial for the article. For this reason, they have been moved to Supplementary Figure 1 along with the experiments using LactA.

The independence of filamentous actin was new to me, but is not relevant for the rest of the paper.

As we said above, we agree with the reviewer that this information is not crucial for the paper. We performed these experiments to address the possibility of actin cables being needed for centromere-SPB association as described in *S. cerevisiae* for telomeres-SPB associations during meiotic prophase (Trelles-Sticken et al., 2005). The results have been moved to Supplementary Figure 1.

Figure 3 describes the search for a mutant combination that leads to prominent dissociation of kinetochores from the SPB region in interphase without impairing viability too much, and the authors successfully identify a useful combination (sad1.2 csi1D).
In Figure 4, the authors then show that the inner kinetochore protein Cnp20 stays localized on kinetochores in interphase in this mutant, but Ndc80 and Nuf2 (Fig. S1) do not. To me, it is only this last piece of information that provides some new insight - and the authors only check three out of many kinetochore proteins.

Thanks for the comment. We have added Mis12 and Spc7 to our study as suggested by the reviewer along with the member of the DASH complex, Dad2. Therefore, we have explored the behavior of the representative proteins of the inner and outer kinetochore: Mis6, Cnp20, Mis12, Ndc80, Nuf2, Spc7 and Dad2. These analyzes have been added in Figures 2, 3 and 4 and in Supplementary Figure 2.

I have some obvious and easy to address questions that - when answered - would make this paper more informative:
For one, I think the idea that what is seen here is 'metazoan-type' assembly/disassembly needs strengthening. For example, do the Mis12 complex and Spc7 (KNL1 ortholog) dissociate from kinetochores as well? Is there any evidence that the regulation could be similar? This could include a sequence analysis for conserved regions, or motifs, or phosphorylation sites.

See our previous answer please. We have found that Mis12 is not disassembled in *sad1.2 csi1*Δ cells, but Spc7 dissociates from the *sad1.2 csi1*Δ kinetochores in a manner similar to Ndc80 and Nuf2 (see

Supplementary Figure 2 and Figure 4). However, the reassembly of Spc7 in the kinetochores is delayed compared to Nuf2 and Ndc80, suggesting the presence of an ordered reassembly sequence likely initiated by Ndc80. This idea has been discussied in the text (lines 466-472).

To better understand the mechanism, I would like to know about the situation where some kinetochores dissociate and some stay associated. Is the outer kinetochore only lost on the dissociating kinetochores? Is the signal loss likely to be cause or consequence of dissociation or unrelated to dissociation?

Thank you for giving us the opportunity to clarify this important point. We have constructed strains that harbor Mis6-mCherry (inner KT) and Ndc80-GFP (outer KT) on *wt* and *sad1.2 csi1*Δ backgrounds. Using these strains, we have verified that only the centromeres dissociated from the SPB lost Ndc80 signal (yellow arrow in Supplementary Figure 3B). These observations suggest that the combination of *sad1.2* mutation and *csi1* deletion could destabilize the outer kinetochore and consequently dissociate the centromeres from the SPB. Also, we performed western blot experiments to gain further insights about this point and we obtained that Ndc80 protein levels are reduced in *sad1.2 csi1*Δ cells compared to *wt* settings during interphase (Figure 4A).

In Figure 4E, the authors do not find any interphase Ndc80 loss in single sad1.2 or csi1D mutants, although about half of these cells do show partial kinetochore dissociation from the SPB region. Does this mean that there is no correlation between dissociation and loss of outer kinetochore proteins?

Consistent with the answer above, there is a correlation between loss of Ndc80 and dissociation in *sad1.2 csi1Δ* settings. The double mutation of *sad1.2* and *csi1Δ* is necessary to produce a clear loss of the outer kinetochore and the total centromere-SPB dissociation. In the single mutants, we found a clear signal of Ndc80 and Nuf2 at the SPB that probably prevents total dissociation of the centromeres (Figure 1G-I). We hypothesize that the amount of the outer kinetochore at the SPB in the single mutants is slightly reduced, leading to partial centromere-SPB dissociation. However, because we are more interested in the reassembly process at mitotic onset, we have focused our work on the analysis of the double mutant since they present important defects in the loss of the outer kinetochore.

What about those sad1.2 csi1D cells where partial kinetochore dissociation occurs? Do all kinetochores lose signal? Or only those that dissociate? Does Ndc80 disappear before or after dissociation?

As we show in Supplementary Figure 3A, in those cases (about 20%) of *sad1.2 csi1Δ* cells that show the Ndc80-GFP signal, the signal is located with the centromeres associated with SPB and the dissociated centromeres lose Ndc80 (as shown in Supplementary Figure 3B). We think that Ndc80 disappears before dissociation.

What about re-tethering experiments, such as those performed in Fernández-Álvarez et al. 2016? Does that rescue Ndc80 signals in interphase in sad1.2 csi1D cells?
It seems such question would be easily addressable by the live-cell imaging approach that the authors have established.

Thanks a lot for the suggestion. We have performed the re-tethering experiments using the GBP-GFP approach and following a similar strategy to the previous work (Fernández-Álvarez et al.,

2016). We found that *sad1.2*-GBP *csi1*Δ Ndc80-GFP cells stabilizes Ndc80 at the centromeres throughout interphase compared to the *sad1.2 csi1*Δ mutant. This control experiment and the quantification of the signal throughout the cell cycle has been added to the new version of the paper (Figure 3C). Moreover, re-tethering experiments using the GBP-GFP approach was used to study if the rescue of Ndc80 signal could also rescue the sensitivity of *sad1.2 csi1*Δ cells to microtubules-depolymerizing drugs (Supplementary Figure 3C and 3D).

Obviously, those are a lot of questions, but I feel that at least some of them need to be explored to add some substance to this interesting observation.

Thank you for the comment on the relevance of our observation. We are happy to answer any other questions the reviewer needs to clarify.

In addition, to be easily understandable (and publishable), the text of this manuscript needs intensive editing. The phrasing at times is so confusing that it becomes hard to understand the intended meaning, and some statements - in their current form - are scientifically incorrect or at least misleading.

Thanks for the suggestion. We have done an intensive review of the article. In addition, we have revised the text in a professional native English service to improve the language. We apologize for any mistakes made when editing the previous version of the article.

Citations should also be carefully revised to pick the most appropriate ones. Just as one example, around line 117-120, a sentence about the situation in fission yeast is followed by two budding yeast citations.

We apologize for the mistake. We have reviewed the citations throughout the document, including the sentences pointed out by the reviewer. Thanks.

Minor comments:
- In the title it seems like "Rabl" is mistakenly written as "RabI" or "Rab1".

We apologise for the mistake, we have revised that Rabl is correctly spelled in the manuscript.

- "arrangement" does not seem the most appropriate word in the title for what the authors want to report.

Thanks for the suggestion, we have replaced the title to "A metazoan-like kinetochore reassembly mechanism is conserved in yeast mitosis but masked by the Rabl configuration". We think that this new version of the title is more representative of our observations.

- *Figure 4E/F legend mentions Mis6 and Nuf2, although the quantification is purely based on Cnp20 and Ndc80 as far as I can tell.*

To reinforce the conclusions of our observations, in this new version of the article we have performed the quantification of all the kintetochore proteins separately in *wt* cells and with Rabl-configuration deficiency throughtout the cell cycle (Figures 2A, B, D, E; Figure 3A, B, D, E; Figure 4B, C, D, E; and Supplementary Figure 2B and C). Also, we have include a quantification of centromere signal throughout interphase for two representative inner and outer kinetochore proteins (Mis6 and Ndc80, respectively) (Supplementary Figure 3A).

- A maximum projection was used for quantification of fluorescence signals. This will not accurately analyze the total signal. It is appropriate for analyzing distances, but not for analyzing intensities. (In my opinion, an average or sum projection should be used).

We have verified that the behavior is very similar using maximum projection and sum projection. In any case, we have recalculated all the quantifications using sum projection and we have used this last for the quantification of all fluorescence signal from protein analysed along the manuscript in *wt* and Rabl-defiencient strains. We have explained in the Methods section the methodology that is followed to accurately quantify the signals represented.

- *Statistics: Figure 3G-I - At least three independent experiments were performed. It would be more informative to show variability between experiments on these graphs rather than just pooling all data.*

Thanks for the suggestion. We have added the variability between experiments to the graphs and we have analysed statistical significance of differences between strains by Fisher's exact test. These new version of the Figure 3G-I have been renamed as Figure 1G-I in the new manuscript.

- *In Figure 3B, it was unclear to me how the arbitrary units for viability were derived, and I was surprised that lem2D csi1D cells scored so low (and show poor growth in Figure 3C), although colony size in the assay in Figure 3A seems normal.*

We have changed the graph to have a clearer representation of these results, representing percentage of cell viability of strains respect to *wt* viability. In our opinion, the analysis performed in this experiment (now in Figure 1B) is more sensitive than the spot assay which objective in the article is to have a global idea of the sensitivity to MBC in the mutants.

Thank you very much for the constructive suggestions. We hope to satisfy all questions, if you have any other suggestions, we will be happy to address them.

Reviewer #2 (Remarks to the Author):

Unlike the inner kinetochore, the metazoan outer kinetochore complex is assembled before mitotic onset and then disassembled after mitosis. By contrast, yeasts constitutively display an outer kinetochore associated to the SPB (centrosome-like structure in fungi). In this ms., Jiménez-Martín and colleagues uncover a metazoan-like process of outer kinetochore recruitment during mitotic onset in fission yeast that is normally masked by the presence of the Rabl configuration. Briefly, the authors designed a genetic system that transiently abrogates Rabl configuration in interphase. This condition triggers outer kinetochore disassembly allowing the detection of outer kinetochore recruitment during mitotic onset. Overall, these observations are unexpected and therefore of outstanding interest for the kinetochore community, not only for fission yeast researchers. I really enjoyed reading this paper and I think that the ms. can be improved if the authors address the following comments, which are mostly suggestions and clarifications.

Major comments and suggestions:

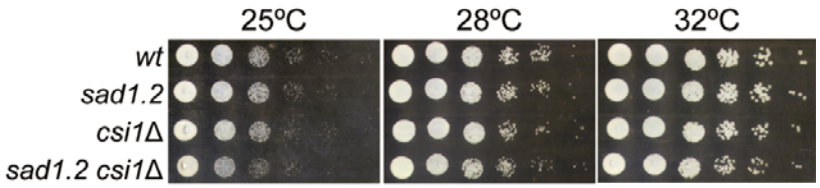
_ It is essential to show data from strains in which outer kinetochore, inner kinetochore and SPB proteins are

all tagged. That would convincingly show that the outer kinetochore disappears when the three centromeres detach from SPB. With the data provided, this can only be assumed.

Thanks for the suggestion, we have built a strain that harbors Ndc80-GFP (outer KT), Mis6-mCherry (inner KT), and SPB in turquoise. As we have answered for reviewer 1, we found that specifically those centromeres dissociated from the SPB lose Ndc80 signal but they keep Mis6. In contrast, the centromere associated with SPB maintains both proteins (see Supplementary Figure 3B). Thanks again for this suggestion that reinforces our observations.

Is the data shown in Figs. 3, 4 and S1 obtained from expts. performed at 32C? It looks like live-cell imaging experiments were performed at 27C according to the Methods. If so, I assume that the double mutant sad1.2 csi1Δ is a hypomorph. A thorough characterization of this mutant would clarify this issue, e.g. dilution assays and liquid growth curves at different temperatures.

Thanks for spotting the error. All experiments of the work are carried out at 32 ° C. We have corrected the error in the Methods section. However, we had previously tested the behavior of the *sad1.2 csi1*Δ mutant at different temperatures. We did not find any significant growth change or defects between 25ºC and 32ºC. See below please.



How about Mis12, KNL-1 or other outer kinetochore proteins that are not in the Ndc80 complex? I assume that such proteins would also disappear when the Ndc80 signal is gone as occurs during meiotic prophase I (Asakawa et al., 2005; Hiraoka group).

Thanks for the suggestion. See reviewer 1's answer, please. We have added three new kinetochore proteins to our analysis: Mis12, Spc7, and Dad2, corresponding to representative members of Mis12 complex, NMS complex and DASH complex, respectively. We found that Spc7 also dissociates from the centromeres and returns before mitosis in Rabl configuration-deficient cells as members analysed from the Ndc80 complex. As suggested by the reviewer, the dynamics of kinetochore reconstruction is very similar to the program in meiosis. We discuss the connections between the two programs on lines 472-488.

Related to above: Hayashi et al. 2006 (Hiraoka group) also showed that outer kinetochore proteins are delocalized but not degraded during meiotic prophase I. I wonder if this also the case for sad1.2 csi1Δ. Western blots for outer kinetochore proteins in interphase would be feasible to perform.

Thanks for the suggestion. We have performed the analysis of Ndc80 protein levels as representative member of the Ndc80 complex and, in contrast to the situation in meiosis, we have seen a downregulation of protein levels of Ndc80 in the *sad1.2 csi1Δ* mutant when cells were enriched in G1 (see Figure 4A, please). Thus, our experiments suggest that Ndc80 protein is degraded and not delocalized in our scenario.

It would be nice to show the recruitment of the outer kinetochore in sad1.2 csi1Δ during mitosis with another approach (preferentially with a non live-cell imaging technique such as ChIP). Perhaps using cut9 or nda3-KM311 mutants may be a way do so.

According to the western blot data (showing reduction of Ndc80 levels in *sad1.2 csi1Δ* cells enriched in G1 (interphase), Figure 4A) together with the observation that specifically the centromeres dissociated from the SPB miss the Ndc80 signal (Supplementary Figure 3A), we hypothetised that ChIP analysis of Ndc80 in *sad1.2 csi1Δ* settings would show similar levels than *wt* cells due to those molecules of Ndc80 in the mutant would be associated to the centromeres.

Strain list must be provided.

We apologize for this error. The list of strains has been added as Supplementary Table 1.

Supp. Figure 1: The GFP dot intensities in sad1.2 csi1Δ sees to be consistently higher than those in wt cells. I wonder if this is something related to photobleaching, or if not, this may be a meaningful observation. In any case, please provide an explanation in the figure legend or text.

Thanks for the comment. We have quantified all the kinetochore protein signals in the *wt* and *sad1.2 csi1Δ* settings considering several points from the background to reduce the influence of photobleaching. The details of the followed methodology are now explained in the Methods section. In general, in this work, we compare the behavior of the genotypes individually, a quantitative comparison between different genotypes has not been studied due to the difficulty of ruling out possible differences in data acquisition.

Minor issues:

Title: Contains "Rabi" instead of "Rabl"

We apologize for the error, we have replaced it with Rabl

Introduction: Please cite Mizuguchi et al. papers (PMID: 25307058 and 26096785) when describing the Rabl configuration in fission yeast.

We have added thesereferences to the introduction. Thanks for the suggestion.

Fig. 1: For some proteins, I notice a slight decrease of the GFP signal as the cells enter mitosis. Can this observation be explained by photobleaching?

We have performed all the quantifications of GFP signals for each protein and normalized them by SPB, this means that during mitosis, some signal decreases not for photobleaching because the amount of protein is distributed between two SPBs.

2F: Please acknowledge the reference in the figure legend (Fernandez-Alvarez and Cooper 2017b). That would help the reader.

Thanks, we have added this reference to the legend of the new Supplementary Figure 1F.

Fig. 3A. I would call it a "strong negative genetic interaction" rather than "synthetic lethality" as some cells are still viable. In fact, the authors state "sporadically sad1.2 lem2Δ csi1Δ....". Could they add more quantification or show more tetrads?

We have added more quantifications from these analyzes and the data has been incorporated in the text (lines 248-250). In addition, we have replaced "synthetic lethality" with "strong negative genetic interaction". We agree with the reviewer that this term is more appropriate for our case.

Fig. 3B: It is not obvious what the assay actually measures unless one reads the Materials section. In any case, OD600 liquid growth curves would be more informative.

We have replaced the previous plot with another plot that we hope will be more intuitive with the experiments performed. Thanks for the suggestion.

Fig. 4. E-F. Line 505 "Quantification of the centromere signals" may be better replaced with "Quantification of cells displaying centromeric signals".

We have replaced the label of these quantifications as the rewiever suggested. Thanks.

Again, we thank the two reviewers for their time, care with the manuscript and suggestions, which we think have led to remarkable improvements in the paper. As a substantial effort have been put into the rewriting of the manuscript and figures, we hope you will now find it suitable for publication in *Molecular Biology of the Cell* and look forward to hearing from you.

We would be glad to respond to any further questions and comments that you may have,

The authors

RE: Manuscript #E20-09-0600R-A
TITLE: "A metazoan-like kinetochore reassembly mechanism is conserved in yeast mitosis but masked by the Rabl configuration"

Dear Dr. Fernández-Álvarez,

Thank you for sending this interesting manuscript back to the Molecular Biology of the Cell. You will see that the reviewers found the manuscript to be considerably improved. As such, it is acceptable in principal. There remain several issues raised by the reviewers that will clarify some issues and strengthen the overall story. In particular, reviewer #1 has significant concerns about Fig. 4, as does reviewer #2 (Fig. 4A and D). There were comments in regard to Fig S2A and S3B (quantitation) that I'm sure you will be able to address. While I agree with the comments regarding Chip strengthing, if this is out of your ability I do not see that acceptance is contingent on this additional experiment.

I look forward to a revised manuscript that addresses these and the additional concerns of the reviewers. Thank you again for this submission.

Sincerely,
Kerry Bloom
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Fernández-Álvarez,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript requires minor revisions before it can be published in Molecular Biology of the Cell, as described in the Monitoring Editor's decision letter above and the reviewer comments (if any) below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you have opted out of publishing the review history.

Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us immediately at mboc@ascb.org.

In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised version, and figures, please use this link (please enable cookies, or cut and paste URL): Link Not Available

Authors of Articles and Brief Communications whose manuscripts have returned for minor revision ("revise only") are encouraged to create a short video abstract to accompany their article when it is published. These video abstracts, known as Science Sketches, are up to 2 minutes long and will be published on YouTube and then embedded in the article abstract. Science Sketch Editors on the MBoC Editorial Board will provide guidance as you prepare your video. Information about how to prepare and submit a video abstract is available at www.molbiolcell.org/science-sketches. Please contact mboc@ascb.org if you are interested in creating a Science Sketch.

Thank you for submitting your manuscript to Molecular Biology of the Cell. Please do not hesitate to contact this office if you have any questions.

Sincerely,

Eric Baker

Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

Jimenez-Martin et al. have submitted a revised version of their paper reporting disassembly of the outer kinetochore in fission yeast interphase when the association between centromeres/kinetochores and the nuclear envelope region underneath the SPB (Rabl configuration) is disrupted.

The representation of the results and the writing has been much improved, and additional outer kinetochore proteins have been examined. The authors have also added a tethering experiment, which showed that artificially recruiting the outer kinetochore protein Ndc80 to Sad1 rescued Ndc80 interphase localization and growth in the sad1-2 csi1D background. The Mis6/Ndc80 co-localization experiment, shown in Figure S3B, is also a great addition. This would become even more valuable by adding a quantification (how often do Mis6 and Ndc80 co-localize at the SPB and away from the SPB), and could then be moved to the main figure.

The authors want to claim that the observed changes in the localization of kinetochore proteins in the sad1-2 csi1D mutant are homologous to metazoan kinetochore disassembly in interphase and reassembly in mitosis. But the basis for this is still very thin. In my opinion, this would require some evidence that similar molecular pathways are involved, which is not examined in this paper.
I therefore think that "metazoan-like" in the title needs to be removed. The authors can speculate on this, and it makes for an adequate hypothesis, but I don't think it's a valid conclusion at this point.

A potential rephrasing of the title could be "Disrupting the Rabl configuration leads to outer kinetochore disassembly during interphase in fission yeast".

On the experimental side: the tethering experiment would be strengthened by showing that centromeres/kinetochores (and not only Ndc80) are recruited. I strongly assume this is the case, since growth is rescued, but it is not formally shown. It could be shown by using Mis6 with a red fluorescent protein, for example. Vice versa, it would be interesting to demonstrate that Ndc80 remains at the kinetochore if tethering is done through Mis6 (or another inner kinetochore protein). But those experiments may be beyond the scope of the current paper.

The other section where I find that conclusions are not fully supported by the data is that on cause and consequences of the delocalization of Ndc80, Nuf2, and Spc7. The authors speculate that "the loss of Csi1 or/and Sad1.2 might destabilize the outer kinetochore, Ndc80 and Nuf2, and thus debilitate the centromere-SPB association". If that was true, I am not sure the tethering experiment using Ndc80-GFP would work in the way it does. The outer kinetochore should still be destabilized in the csi1D sad1-2 genetic background - and while Ndc80 would tether, the rest of the kinetochore may not. (As described above, this could be experimentally tested, but the growth-rescue suggests that entire kinetochores/centromeres are co-tethered along with Ndc80-GFP).
I find it more likely that the NE region beneath the SPB provides an environment (maybe kinase activity?) that keeps the kinetochore intact, and loss of that activity when kinetochores are away from that region leads to disassembly. I think the discussion around lines 376 - 380 needs to be changed for the same reason.

I am also not entirely convinced by the experiment shown in Figure 4A. "G2/M" is not a good description of a cell cycle phase, and the methods section in fact suggests that the "G2/M" extract was made from asynchronously growing cells (which in fission yeast would be mostly cells in G2, with some S and M phase mixed in). Hence, these cells should actually correspond to what is seen by microscopy (G2 and early M), which would suggest that Ndc80 dissociates, but is not degraded. The G1 enrichment is done by nitrogen depletion, which sends the cells on the path to sexual differentiation, and is not a typical somatic cell cycle G1 situation. Typical methods of G1 enrichment would be elutriation, sucrose gradient centrifugation, cdc10 mutation, or release from a mitotic arrest, e.g. by nda3-KM311.
On the purely technical side, it would be better to quantify the two experiments that have been performed and show this quantification in addition to the semi-quantitative data that are shown.

Other minor comments:

In line 327-334, the authors discuss possible reasons for the decrease in Mis6-GFP signal observed in mitosis in wt cells. I can't quite follow the argument. I don't understand what is meant by "being allocated to the two SPBs". The splitting of centromeres in mitosis shouldn't influence how much total signal is recorded. And I am not aware that Mis6 re-localizes to SPBs in mitosis, but maybe it does? I also don't see any evidence that kinetochore proteins "accumulate" in the sad1-2 csi1D cells - shouldn't this lead to an increase in total localized signal (which is not obvious in Fig. 2B)?

Methods:
• Line 502: Information on how many cells were plated would be useful.
• Lines 633 - 640: How the OD / cell concentration normalization works is not clear to me.
• Line 687: isn't 15 µL of a 1 mM stock in 3 mL a concentration of 5 µM rather than 6 µM?
• Lines 521, 536, and 550 are a little unclear. More than 10 cells in all experiments together, or in each experiment? Why "> 10 cells" when the figure shows n = 10 or lower.
• Line 713-714: It is unclear how G2/M enrichment was performed. This reads like asynchronously growing cultures were used.

References:
• Line 85 lacks a reference for the dismantling of the Rabl configuration in metazoans, I think.
• Line 126: I may be misunderstanding, but I don't see that topic discussed in the Gu/Oliferenko paper that is cited.

Writing:
• Line 212: I'd prefer "leads to cell lethality" rather than "total cell lethality" - since I don't think partial lethality is possible.
• Line 245/246. It is unclear to me how this is supported by the data presented. And I don't think the sentence is needed to argue why sad1.2 csi1D double mutants are examined.
• Line 374: "confirmed that ... levels are reduced". I don't think "confirm" is the right word - since whole cell concentration has not been assessed in the microscopy data. Hence, the microscopy data would also be consistent with mere delocalization, not necessarily degradation.
• The title of Supplementary Figure 1 does not capture the content of the figure well, since only one strategy was successful. Maybe "Probing requirements for the Rabl configuration in fission yeast" or something along these lines.
• Line 360 "throughout the cell cycle"; line 500: should be "spores harbor"; ine 556: "In metazoans,..." or "In metazoan cells,..."; line 576: "is shown"; line 596: "were quantified" or "intensity was..."; lines 634/641/682: "grown to..."; line 645: "normalized to the colony number of wt cells"; line 662: delete "respect"; line 670: shouldn't this be "... foci are all those outside fo the SBP co-localization area"?; line 673: "for each satellite focus"; line 717: "stored"
• Line 485 - 489: I don't understand what the sentence is trying to say. Since it's in the discussion part, it's not a problem - but maybe the authors want to revise to make their point clear.
• Lines 524 and 537/538 should probably be "just before spindle formation" or "just before SPB separation".

Figures:
• Figure 5: I think the figure would be easier to understand if the text "Outer Kinetochore Assembly" and "... Disassembly" as well as "Ndc80/Nuf2/Spc7 Assembly" and "... Disassembly" was in black rather than green. (Since green is the color used for SPBs - whereas grey/black is used for kinetochores.) The text "Constitutive ..." in the center could be in grey to match the color in the schematic.
• Figure S2A: The current version seems to imply that Spc7 bridges the Ndc80 and Mis12 complexes, which is most likely not the case. I'm also not sure how well the inner kinetochore arrangement represents current knowledge. Maybe just listing components of the outer and inner kinetochore would be sufficient.

Reviewer #2 (Remarks to the Author):

The manuscript has been considerably improved. I appreciate that the authors addressed most of my concerns. However, given that the claims and implications of this study are very important for the kinetochore community, I think that it is still essential to demonstrate that the described phenomenon can also be observed by employing another approach such as ChIP using a less bulky tag (i.e. HA instead of GFP). That would rule out any hypothetical artifact related to the tagging of proteins. Indeed, the authors now show that tagging Mis12 with GFP has a suppressor effect. The ChIP experiment that this reviewer proposes is very feasible. In interphase, one would expect lower enrichment of Ndc80/Nuf2 at centromeres compared to WT. Conversely, in mitosis Ndc80/Nuf2 enrichment would increase to WT levels.

Apart from that, I have some minor comments as follows:
_ Fig 1A: The thickness of the geometrical shapes does not allow for full appreciation of the colony size.
_ Fig. 1B: At first sight, this set of data is somewhat confusing when compared to Fig. 1A. For instances, lem2Δ csi1Δ seems to be healthy in the tetrad plates but not in Fig. 1B. In any case, a schematic of the experiment performed in Fig. 1B would clarify that the experiments performed are unrelated.
_ Fig. 2C and 2F. It is unclear the N of cells that are used in this graph.
_ Fig. S2A: The kinetochore schematic can be improved as many fundamental proteins are missing (e.g. Cnp3/CENP-C). Perhaps the authors could show only the subcomplexes and include the names of the relevant proteins for this study (e.g. Cnp20, Mis12, etc). I understand that the authors modified a figure from Hayashi et al., 2006. It would be helpful to use an updated schematic from more recent literature. In addition, human names in superscript will be useful for a broad audience.
_ Fig. S3C. This needs quantification. At least, display percentage of cells that exhibit declustering.
_ Fig. 4A: "-" must read "untagged".
_ Fig. 4D. Although interesting, experiments performed with the DASH complex may be difficult to interpret as these proteins are by default absent in interphase. Hence, I wonder how the authors know which of the analyzed cells display "transient total

centromere dissociation". One could assume ~30% based on Fig. 1H but obviously a double tag (i.e. Mis6 and Dad2) would be unambiguous. In any case, this technical issue must be acknowledged.

Dear Editor,

We hereby submit a revised version of the manuscript E20-09-0600-R, entitled "*The Rabl chromosome configuration masks a kinetochore reassembly mechanism in yeast mitosis*". We are grateful for the comments made by the reviewers, and we thank them for their constructive suggestions. We have addressed all reviewer comments, including the ChIP experiments suggested by reviewer 2, and in doing so have clarified and strengthened our results. We hope that this latest version of our work is suitable for publication in Molecular Biology of the Cell.

Here, we respond to each of the reviewer comments in turn:

Reviewer #1 (Remarks to the Author):

Jimenez-Martin et al. have submitted a revised version of their paper reporting disassembly of the outer kinetochore in fission yeast interphase when the association between centromeres/kinetochores and the nuclear envelope region underneath the SPB (Rabl configuration) is disrupted.

The representation of the results and the writing has been much improved, and additional outer kinetochore proteins have been examined. The authors have also added a tethering experiment, which showed that artificially recruiting the outer kinetochore protein Ndc80 to Sad1 rescued Ndc80 interphase localization and growth in the sad1-2 csi1D background. The Mis6/Ndc80 co-localization experiment, shown in Figure S3B, is also a great addition. This would become even more valuable by adding a quantification (how often do Mis6 and Ndc80 co-localize at the SPB and away from the SPB) and could then be moved to the main figure.

Thanks for the nice comments on the improvement of the paper. Initially, we had not included the quantification of the cases where Mis6 and Ndc80 co-localize far from the SPB in *sad1.2 csi1Δ* settings because they are very rare. We have now included these quantifications in Supplementary Fig. 3B. Hence, we found a tight correlation between centromeres dissociated from the SPB and the loss of Ndc80 signal. However, although we agree with the reviewer about the relevance of these data, we are inclined to keep these experiments in Supplementary Figure 3B as, in our opinion, they rather represent control experiments supporting the Figure 3.

The authors want to claim that the observed changes in the localization of kinetochore proteins in the sad1-2 csi1D mutant are homologous to metazoan kinetochore disassembly in interphase and reassembly in mitosis. But the basis for this is still very thin. In my opinion, this would require some evidence that similar molecular pathways are involved, which is not examined in this paper. I therefore think that "metazoan-like" in the title needs to be removed. The authors can speculate on this, and it makes for an adequate hypothesis, but I don't think it's a valid conclusion at this point. A potential rephrasing of the title could be "Disrupting the Rabl configuration leads to outer kinetochore disassembly during interphase in fission yeast".

Following the reviewer's suggestion, we have reworded the title to "*The Rabl chromosome configuration masks a kinetochore reassembly mechanism in yeast mitosis*." We

believe that this new version of the title harbors the most unexpected result of our work, the presence of the outer KT reassembly mechanism just before mitosis but without the evolutionary implications.

On the experimental side: the tethering experiment would be strengthened by showing that centromeres/kinetochores (and not only Ndc80) are recruited. I strongly assume this is the case, since growth is rescued, but it is not formally shown. It could be shown by using Mis6 with a red fluorescent protein, for example. Vice versa, it would be interesting to demonstrate that Ndc80 remains at the kinetochore if tethering is done through Mis6 (or another inner kinetochore protein). But those experiments may be beyond the scope of the current paper.

Thanks for the comment. We agree with the reviewer that the fact that cells are recovering normal growth on TBZ-containing media is strong evidence that the kinetochore reassembles well enough. We are interested in the future to explore in more detail how the dynamic of outer kinetochore recruitment is established where we will perform tethering experiments in different scenarios.

The other section where I find that conclusions are not fully supported by the data is that on cause and consequences of the delocalization of Ndc80, Nuf2, and Spc7. The authors speculate that "the loss of Csi1 or/and Sad1.2 might destabilize the outer kinetochore, Ndc80 and Nuf2, and thus debilitate the centromere-SPB association". If that was true, I am not sure the tethering experiment using Ndc80-GFP would work in the way it does. The outer kinetochore should still be destabilized in the csi1D sad1-2 genetic background - and while Ndc80 would tether, the rest of the kinetochore may not. (As described above, this could be experimentally tested, but the growth-rescue suggests that entire kinetochores/centromeres are co-tethered along with Ndc80-GFP).
I find it more likely that the NE region beneath the SPB provides an environment (maybe kinase activity?) that keeps the kinetochore intact, and loss of that activity when kinetochores are away from that region leads to disassembly. I think the discussion around lines 376 - 380 needs to be changed for the same reason.

We think that both hypotheses are not mutually exclusive. Our hypothesis on whether the loss of Csi1 or / and Sad1.2 might destabilize the outer kinetochore is consistent with the Ndc80 tethering experiments since, in our opinion, the "forced" recruitment of Ndc80 to the SPB might also stabilize Nuf2, Spc7, Mis12, proteins with which Ndc80 shows physical interaction. However, we agree with the reviewer on the possibility that the SPB might provide a micro-environment that maintains the identity of the centromere (also recently suggested by Allshire lab, bioRxiv 2021.12.16.473016; doi: https://doi.org/10.1101/2021.12.16.473016) and the stability of the kinetochore. We have included this possibility on lines 387-391 in the revised version of the paper.

I am also not entirely convinced by the experiment shown in Figure 4A. "G2/M" is not a good description of a cell cycle phase, and the methods section in fact suggests that the "G2/M" extract was made from asynchronously growing cells (which in fission yeast would be mostly cells in G2, with some S and M phase mixed in). Hence, these cells should actually correspond to what is seen by microscopy (G2 and early M), which would suggest that Ndc80 dissociates,

but is not degraded. The G1 enrichment is done by nitrogen depletion, which sends the cells on the path to sexual differentiation, and is not a typical somatic cell cycle G1 situation. Typical methods of G1 enrichment would be elutriation, sucrose gradient centrifugation, cdc10 mutation, or release from a mitotic arrest, e.g. by nda3-KM311.

On the purely technical side, it would be better to quantify the two experiments that have been performed and show this quantification in addition to the semi-quantitative data that are shown.

We have constructed new strains using the *cdc25-22* and the *cdc10-129* thermosensitive alleles to enrich the cultures in G2/M and G1, respectively, as suggested by the reviewer. Also, we have tagged Ndc80 with an HA epitope for western blot and ChIP experiments as the reviewer #2 suggested. The results of the western analysis are shown in the new version of Figure 4A. Furthermore, we show in the figure the quantification from two independent experiments. Congruently to our previous observations, G1-enriched cultures show a significant reduction in the amount of Ndc80 in *csi1Δ sad1.2* mutants.

Other minor comments:

In line 327-334, the authors discuss possible reasons for the decrease in Mis6-GFP signal observed in mitosis in wt cells. I can't quite follow the argument. I don't understand what is meant by "being allocated to the two SPBs". The splitting of centromeres in mitosis shouldn't influence how much total signal is recorded. And I am not aware that Mis6 re-localizes to SPBs in mitosis, but maybe it does? I also don't see any evidence that kinetochore proteins "accumulate" in the sad1-2 csi1D cells - shouldn't this lead to an increase in total localized signal (which is not obvious in Fig. 2B)?

Thank you for giving us the opportunity to clarify this point. The signal is represented by SPB, with time 0 being the first frame showing two SPBs. This means that at time 10 min, for example, the total amount per cell is approximately the double of the intensity displayed. In the case of the *sad1.2 csi1Δ* mutant, the signal is stable throughout the analysis, suggesting that the signal increases during mitosis. In any case, we consider that these observations are not related to the main idea of the article, so we have eliminated these lines as they are mostly speculative.

Methods:

- *Line 502: Information on how many cells were plated would be useful.*

300 cells were plated. The information has been added on line 502

- *Lines 633 - 640: How the OD / cell concentration normalization works is not clear to me.*

We have added the cell concentration before normalization on line 646

- *Line 687: isn't 15 µL of a 1 mM stock in 3 mL a concentration of 5 µM rather than 6 µM?*

We apologize for the mistake. It's 5 μM as the reviewer says.

• Lines 521, 536, and 550 are a little unclear. More than 10 cells in all experiments together, or in each experiment? Why "> 10 cells" when the figure shows n = 10 or lower.

We apologize for the confusion. The plot represents the dynamics of exactly 10 cells. Initially, we wanted to include the number of cells showing signal per time point since, for example, not all cells recover the Ndc80 signal at the same time point, but we find this confusing and not intuitive to visualize, so we have simplified the graphs and legends in the new version of the figures.

• Line 713-714: It is unclear how G2/M enrichment was performed. This reads like asynchronously growing cultures were used.

As said above, we have changed the genetic background for the G1 and G2/M enrichment experiments using the *cdc10-129* and *cdc25-22* thermosensitive alleles, respectively. A new method section has been added in the revised version that includes the protocol followed for these new experiments ("Cell cycle synchronizations in G1 and G2/M").

References:
• Line 85 lacks a reference for the dismantling of the Rabl configuration in metazoans, I think.
• Line 126: I may be misunderstanding, but I don't see that topic discussed in the Gu/Oliferenko paper that is cited.

Both suggestions about the references have been addressed. Thanks.

Writing:
• Line 212: I'd prefer "leads to cell lethality" rather than "total cell lethality" - since I don't think partial lethality is possible.
Line 212: we have replaced to "leads to cell lethality"

• Line 245/246. It is unclear to me how this is supported by the data presented. And I don't think the sentence is needed to argue why sad1.2 csi1D double mutants are examined.

We have rephrased these lines following her/his suggestion (lines 245-249).

• Line 374: "confirmed that ... levels are reduced". I don't think "confirm" is the right word - since whole cell concentration has not been assessed in the microscopy data. Hence, the microscopy data would also be consistent with mere delocalization, not necessarily degradation.

We have removed the word "confirm" from this sentence. Thanks for the suggestion.

• The title of Supplementary Figure 1 does not capture the content of the figure well, since only one strategy was successful. Maybe "Probing requirements for the Rabl configuration in fission yeast" or something along these lines.

We have replaced the title of Supplementary Figure 1 as the reviewer suggests.

• Line 360 "throughout the cell cycle"; line 500: should be "spores harbor"; ine 556: "In metazoans,..." or "In metazoan cells,..."; line 576: "is shown"; line 596: "were quantified" or "intensity was..."; lines 634/641/682: "grown to..."; line 645: "normalized to the colony number of wt cells"; line 662: delete "respect"; line 670: shouldn't this be "... foci are all those outside fo the SBP co-localization area"?; line 673: "for each satellite focus"; line 717: "stored"

Thanks for all the suggestions. We have addressed all these issues as suggested.

• Line 485 - 489: I don't understand what the sentence is trying to say. Since it's in the discussion part, it's not a problem - but maybe the authors want to revise to make their point clear.

We have removed these speculative lines since they are not a crucial point for the discussion of the results. Thanks.

• Lines 524 and 537/538 should probably be "just before spindle formation" or "just before SPB separation".

We apologize for the issue, the right is to say: "just before SPB separation".

Figures:
• Figure 5: I think the figure would be easier to understand if the text "Outer Kinetochore Assembly" and "... Disassembly" as well as "Ndc80/Nuf2/Spc7 Assembly" and "... Disassembly" was in black rather than green. (Since green is the color used for SPBs – whereas grey/black is used for kinetochores.) The text "Constitutive ..." in the center could be in grey to match the color in the schematic.
Thanks for the useful comments to improve the model. We have followed all the suggestions.

• Figure S2A: The current version seems to imply that Spc7 bridges the Ndc80 and Mis12 complexes, which is most likely not the case. I'm also not sure how well the inner kinetochore arrangement represents current knowledge. Maybe just listing components of the outer and inner kinetochore would be sufficient.

We have added a new version of the schematic following the suggestions from both reviewers. Thanks.

Reviewer #2 (Remarks to the Author):

The manuscript has been considerably improved. I appreciate that the authors addressed most of my concerns. However, given that the claims and implications of this study are very important for the kinetochore community, I think that it is still essential to demonstrate that the described phenomenon can also be observed by employing another approach such as ChIP using a less bulky tag (i.e. HA instead of GFP). That would rule out any hypothetical artifact related to the tagging of proteins. Indeed, the authors now show that tagging Mis12 with GFP has a suppressor effect. The ChIP experiment that this reviewer proposes is very feasible. In interphase, one would expect lower enrichment of Ndc80/Nuf2 at centromeres compared to WT. Conversely, in mitosis Ndc80/Nuf2 enrichment would increase to WT levels.

Thank you for the nice comments on the improvement of the paper and the relevance of our observations. To strengthen our observations, we have confirmed our experiments by ChIP as suggested. We have constructed new strains using HA instead of GFP for Ndc80 tagging on a *cdc10-129* and *cdc25-22* backgrounds to enhance the enrichment of cultures in G1 and G2/M, respectively. Western blot analysis confirmed our previous observations that Ndc80 protein levels in G1 are reduced in *sad1.2 csi1Δ* mutants. Moreover, our ChIP-qPCR analysis in G1 shows a severe reduction in the enrichment of Ndc80 at centromeres 1 and 2 compared to wt conditions. We agree with the reviewer that this complementary approach strengthens our results. Thanks.

Apart from that, I have some minor comments as follows:
_ Fig 1A: The thickness of the geometrical shapes does not allow for full appreciation of the colony size.

Thanks for the suggestion which it has been addressed.

_ Fig. 1B: At first sight, this set of data is somewhat confusing when compared to Fig. 1A. For instances, lem2Δ csi1Δ seems to be healthy in the tetrad plates but not in Fig. 1B. In any case, a schematic of the experiment performed in Fig. 1B would clarify that the experiments performed are unrelated.
We have added a schematic of the experiment in 1B to avoid confusion, as the experiments are not related, with 1B being more sensitive to detect growth defects. Thanks for the suggestion.

_ Fig. 2C and 2F. It is unclear the N of cells that are used in this graph.

See the response to reviewer 1, please. The number of cells scored is 10 throughout the experiments. We initially added the number of events per timepoint per analysis, but we have removed that information in the last version to avoid misunderstanding.

_ Fig. S2A: The kinetochore schematic can be improved as many fundamental proteins are missing (e.g. Cnp3/CENP-C). Perhaps the authors could show only the subcomplexes and include the names of the relevant proteins for this study (e.g. Cnp20, Mis12, etc). I understand that the authors modified a figure from Hayashi et al., 2006. It would be helpful to use an

updated schematic from more recent literature. In addition, human names in superscript will be useful for a broad audience.

We have simplified the schematic, added some missing proteins and the name of orthologs in humans following his/her suggestions. Thanks.

_ Fig. S3C. This needs quantification. At least, display percentage of cells that exhibit declustering.

We have added the quantification to Fig. S3B. Please, see reviewer #1 comments.

_ Fig. 4A: "-" must read "untagged".

We have modified this panel as suggested and we have included the new experiments performed using *cdc25-22* and *cdc10-129* strains.

_ Fig. 4D. Although interesting, experiments performed with the DASH complex may be difficult to interpret as these proteins are by default absent in interphase. Hence, I wonder how the authors know which of the analyzed cells display "transient total centromere dissociation". One could assume ~30% based on Fig. 1H but obviously a double tag (i.e. Mis6 and Dad2) would be unambiguous. In any case, this technical issue must be acknowledged.

We checked that the penetrance of the centromere dissociation in interphase is not altered by Dad2-GFP tagging using strains harboring Mis6-mCherry and Sad1.2-Turquoise. However, we decided to include in the manuscript only the analysis performed using the strains harboring Sid4-mCherry, mCherry-Atb2 and Dad2-GFP to be consistent with all the previous quantifications in Figure 2 and 3. The penetrance of the centromere dissociation is not altered by the GFP-tagging of Dad2 (74 cells out of 100 showed partial centromere dissociation).

We tried to repeat the entire analysis by *in vivo* microscopy using three colors (blue, red and green) but, in our hand, we cannot maintain a good enough blue (CFP or turquoise) signal for two hours by taking points every 5 minutes.

Again, we thank the reviewers for their time, care with the manuscript and suggestions, which we think have led to remarkable improvements in the paper. As a substantial effort have been put into the rewriting of the manuscript and figures, we hope you will now find it suitable for publication in *Molecular Biology of the Cell* and look forward to hearing from you.

We would be glad to respond to any further questions and comments that you may have,

The authors

RE: Manuscript #E20-09-0600RR
TITLE: "The Rabl chromosome configuration masks a kinetochore reassembly mechanism in yeast mitosis"

Dear Dr. Fernández-Álvarez,

Thank you for your thorough response to the reviewer's comments. I am pleased to inform you that your manuscript is now suitable for publication in the Molecular Biology of the Cell. Congratulations and thank you for submitting this nice work.

Sincerely,
Kerry Bloom
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Fernández-Álvarez:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

Within approximately four weeks you will receive a PDF page proof of your article.

Would you like to see an image related to your accepted manuscript on the cover of MBoC? Please contact the MBoC Editorial Office at mboc@ascb.org to learn how to submit an image.

Authors of Articles and Brief Communications are encouraged to create a short video abstract to accompany their article when it is published. These video abstracts, known as Science Sketches, are up to 2 minutes long and will be published on YouTube and then embedded in the article abstract. Science Sketch Editors on the MBoC Editorial Board will provide guidance as you prepare your video. Information about how to prepare and submit a video abstract is available at www.molbiolcell.org/science-sketches. Please contact mboc@ascb.org if you are interested in creating a Science Sketch.

We are pleased that you chose to publish your work in MBoC.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org
