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PRC1-labeled microtubule bundles and kinetochore pairs show one-to-one association in metaphase

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1st Editorial Decision 01 August 2016

Thank you for the submission of your research manuscript to EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, all referees acknowledge the interest of the findings and support publication of the study after revisions. However, all referees have raised several concerns that I ask you to appropriately and fully address in a revised version. In particular, please outline in the revised version (also in the discussion - see comments of referee #3) clearer the reasoning behind the experimental approaches and why this manuscript expands significantly on your previous paper.

All referee concerns (as detailed in their reports) must also be fully addressed in a complete point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further.

REFEREE REPORTS

Referee #1:

The main finding reported in this manuscript is that most centromeres are adjacent to a PRC1-decorated bundle of non-kinetochore microtubules during metaphase in human cells. The presence of the PRC1 bundles is necessary for proper stretching of the centromeres. The interaction between PRC1 bundles and centromeres and its importance for interkinetochore tension have been previously characterized by the same group of investigators (Nature Communication 2016). However, the observation that virtually each centromere interacts with a PRC1 bundle is novel. The conclusions are based primarily on straightforward correlative analyses in cells that either express FP-tagged proteins or are immunostained for the endogenous proteins. This approach is adequate and the experimental data justify the conclusions. The take home message is clear and succinct. There are just a few minor concerns/recommendations that the authors may want to address (listed below). Overall, this is an interesting study that will be appreciated by the scholars of cell division.

- Abstract: "the number of PRC1-GFP-labeled bundles per spindle matches the number of kinetochore pairs." This is a bit of overstatement. The data suggest that ~90% of centromeres interact with PRC fibers but an exact match between the number of fibers and centromeres is not proven.
- Introduction: "Meanwhile, non-kinetochore microtubules extend from opposite spindle poles" - Not all non-kinetochore microtubules extend from the poles. Many are formed within the spindle.
- In the concluding paragraphs authors discussed how stability of the certain parameters such as a length of antiparallel overlap can be conjugated with reduced by PRC1 knockdown interkinetochore tension (Page 12). They also discuss thinner bridging fibers after the knockdown procedure. However, the discussion is not directly linked to their own recently published model (Nature Communications 7, 2016). This model explicitly predicts that the forces at the pole and at the kinetochore depend on the bridging fiber thickness.
- Formation of PRC1 homodimer required for the effective interaction with kinesin-4 and microtubule binding affinity is believed to be triggered at the onset of anaphase. This should be considered in the discussion.
- Fig 1. 3D projections in A and B are a bit confusing because the orientation of the spindle axis is not marked in the individual panels. Reading these panels requires a certain level of expertise in spindle architecture.
- Fig 3C. The white line on the image obscures what exactly was contoured. In fact, it appears that both microtubules and PRC1 bundle arc upwards, not downwards as the schematic shows. It is important to show both the real image and the contour in parallel. Further, contour lines that connect spindle poles may be problematic if the centromere axis (which should be parallel to the PRC1 bundle) is significantly tilted with respect to the spindle axis. Clear examples of these tilted PRC1 bundles are present in Fig.1A. How was this issue dealt with?
- Fig 3E. How exactly is the Lprc1 value determined? The scan is understandably noisy so the exact width of the peak cannot be set arbitrarily. What signal processing routines are applied?
- Expanded view Video 3. The intensity of magenta signal increases abruptly between 143 and 156 seconds. It looks like the acquisition parameters were changed; however, such a change is not reported.
- Fig 4A. The signal/noise ratio is quite different in control vs. PRC1 siRNA. Does this imply that the total amount of tubulin incorporated into spindle (and individual k-fibers) is affected by PRC1 siRNA? Why is the spindle on the right so dim? The reason I bring up this issue is that if the number of microtubules within K-fibers and other bundles decreases upon PRC1 depletion, then the changes in inter-kinetochore distances could be a consequence of the lower number of microtubules, not a direct effect of PRC1 depletion. It may be important to reveal absolute values of Ib/Ibk not just the ratios.

Referee #2:

Polak et al. report on "bridging fibres" of the mitotic spindle. A previous publication from the Toli lab described these fibres, but now this paper proposes that there is a one-to-one association between bridging fibres and sister kinetochore pairs. It's an interesting piece of work and will be of interest to those of us working on spindle structure. Overall, I think the case is good. The three main observations here are:

1. PRC1 decorates antiparallel MTs in a metaphase spindle, which despite intense study has been

overlooked. This is not limited to overexpression of PRC1-GFP as it can be seen by immunofluorescence.
2. The number of bridging fibres correlate with the number of kinetochores in a cell line HeLa (with a variable chromosome number). Indicating a one-to-one relationship.
3. Colocalisation of PRC1-positive spots with kinetochores in live cell imaging experiments.

Two comments:

1. The strongest result in the paper is from the live cell imaging showing that XY coordinates of the PRC1-positive structures co-vary with the kinetochore puncta. The pie chart and plot in EV2B are OK (the plot in EV2A is dispensable), but the analysis could improved here to underline what for me is very strong evidence for a relationship between the bridging fibres and the kinetochores. Examples include: particle image cross-correlation (Schmidt lab, Biophys J 2011), plotting relative XY displacement vectors as a function of time, covariance matrices for the two signals over time, comparison with nearest neighbour versus next nearest neighbour, etc. There are quite a few unbiased methods to look at this. As it is, the plot in EV2B just shows that the pairings don't move too far apart (it is also difficult to make out the individual events). The pie chart summarises the observations against a criterion, but it is a bit "qualitative". I think it is worth the authors putting in a bit more effort with this data, it will improve this part.

2. Despite the correlations being good evidence for a one-to-one relationship, I somehow think it's a bit strong to say that ALL kinetochore pairs are associated with a bridging fibre. This statement leaves no wriggle room for i) unpairing and repairing dynamics between kinetochores and bridging fibres, ii) newly-attached or newly-syntelic kinetochores becoming paired. Both of these things probably happen and so the authors should think about whether it's wise to imply that the relationship is obligatory, i.e. one-to-one. After all, ~12% of PRC1 bundles are kinetochore free and ~6% of kinetochores have no PRC1 associated. There is experimental error, sure, but this is not one-to-one.

Referee #3:

In their most recent paper about bridging microtubules, Polak et al perform a systematic analysis to understand the extent to which the overlap fibers are found in metaphase spindles, and to determine whether each kinetochore pair is indeed associated with such fibers, or whether only a subset of KT pairs are associated with such fibers. Their data indeed supports the notion that there is a one-to-one association between PRC1-labeled MT bundles and kinetochore pairs (as indicated by the title). The current work is thorough and carefully performed.

I only have a few comments below addressing specific concerns, one of which raises the question of whether the proposed function of these fibers (to provide shape to the spindle) is supported by their work. Besides those concerns outlined below, it's unclear whether the current body of work expands significantly on their previous work (their Nature Communications paper), other than to describe the presence of the overlap fibers throughout the spindle (rather than just at the edges). This may be due in part to the fact that throughout much of the manuscript, there is little explanation of the reasoning behind a particular experimental approach. Moreover, the discussion offers little other than a rehashing of the results. For instance, why did the authors look for a relationship between PRC1 signal and distance from the long axis of the spindle? Why did they explore the relationship between chromosome number and spindle length/width? Why did the authors bother to look at endogenous PRC1, rather than focus on episomal PRC1-GFP? What can be surmised from their findings (what does it mean that there is no relationship between PRC1 intensity and distance from spindle long axis?)? Other than describing the ubiquity of the overlap fibers throughout the spindle, I'm not sure what new findings are described in this current manuscript. I hope the comments below help the authors improve the clarity of their manuscript.

Major points:

1) As shown in Fig. 2, the majority of PRC1 labeled bundles indeed appear to move in a similar pattern to that of the associated kinetochore pair. However, could these correlated movements be due to the entire spindle moving as a cohesive unit? As a control, it would be helpful to compare the movement of non-closely associated, randomly chosen PRC bundles and kinetochore pairs.

2) If reducing PRC1 levels by siRNA reduces bridging MT number, and the spindle shape is presumably reliant on these bridge fibers (akin to the wooden rod and rope analogy described in Iva's recent Cell Cycle commentary), then why does the shape of the spindle not change upon PRC1 knock-down (at least as measured from spindle height and width in Table 2)? Maybe I'm missing something, but this seems to be a key feature of bridging MTs: that they provide sufficient rigidity to support the curved shape of the spindle. The authors alluded to something similar in their Nature Communications paper (cells with thicker fibers have a curved shape, while those with thinner fibers have a diamond shape).

3) The authors note a reduction in inter-KT distance upon PRC1 knock-down, and attribute this to a reduction in bridging fiber thickness (i.e., # of MTs making up a fiber). It's unclear to me how reduction in bridging fiber thickness results in reduced inter-KT distance. Given the authors have previously generated a theoretical model of spindles, it would be helpful if they either applied the model to help explain this observation, or discussed data previously acquired to help the reader understand where the force imbalance is (i.e., what is the source) that leads to the reduced inter-KT distances.

Minor points:

1) It is unclear why the authors tested for a correlation between PRC1 signal (or inter-KT distances), and distance from the spindle long axis? Perhaps I'm missing something, but I don't understand why there would be any relationship between these two parameters. This would imply that the further 'up' or 'down' laterally one considers from the long axis (with respect to a bird's eye view of the spindle), there might be a change in the density of PRC1. I understand that, according to EV3, there is no relationship, but isn't this expected? Unless the authors were expecting a relationship between degree of kMT curvature and bridge MT number? In any case, this point needs to be clarified.

2) The following sentence is confusing as worded: "Next, we tested whether the distance between sister kinetochores and spindle proportions is affected by PRC1 siRNA." It sounds like they are testing for distances between 'spindle proportions' as well between sister KTs. The sentence should be reworded.

3) Figure 4E appears to be monochromatic in spite of the label indicating the presence of green (tubulin) and magenta (PRC1). The legend indicates "Only AlexaFluor555 channel is shown," in which case the label above the figure needs to be changed.

4) Similarly, Fig. 4G indicates the presence of green and magenta, but I only see magenta.

5) Also, the plot in Fig. 4F has tubulin-GFP and PRC1-AlexaFluor555 labels above it. Unless these labels are meant for Fig. 4E? The labeling is quite confusing throughout much of Fig. 4 (same confusion in panel H).

6) In contrast, the two-color images in Fig. 2D are unlabeled. I realize the legend indicates what the colors represent, but it would be easier for the reader to see the labels on the figure.

7) The text points to Fig. 4G twice in a row on page 11 ("PRC1-GFP were altered by PRC1 knockdown (Fig 4G, Fig 4G"...)

8) It is unclear how PRC1 "density" was determined, or what that even refers to (Fig. 1G). Are they referring to the size of the fluorescent spot (the units suggest as much)? Or, are they referring to the # of PRC1 spots per unit area? This needs to be clarified, and the text needs to include an explanation of the relevance of this data.

1st Revision - authors' response 21 October 2016

Referee #1:

The main finding reported in this manuscript is that most centromeres are adjacent to a PRC1-decorated bundle of non-kinetochore microtubules during metaphase in human cells. The presence

of the PRC1 bundles is necessary for proper stretching of the centromeres. The interaction between PRC1 bundles and centromeres and its importance for interkinetochore tension have been previously characterized by the same group of investigators (Nature Communication 2016). However, the observation that virtually each centromere interacts with a PRC1 bundle is novel. The conclusions are based primarily on straightforward correlative analyses in cells that either express FP-tagged proteins or are immunostained for the endogenous proteins. This approach is adequate and the experimental data justify the conclusions. The take home message is clear and succinct. There are just a few minor concerns/recommendations that the authors may want to address (listed below). Overall, this is an interesting study that will be appreciated by the scholars of cell division.

1) Abstract: "the number of PRC1-GFP-labeled bundles per spindle matches the number of kinetochore pairs." This is a bit of overstatement. The data suggest that ~90% of centromeres interact with PRC fibers but an exact match between the number of fibers and centromeres is not proven.

Authors: We agree with the reviewer. A similar issue was raised by Referee 2 (point 2). We changed the quoted sentence into: "We found that the number of PRC1-GFP-labeled bundles per spindle is nearly the same as the number of kinetochore pairs."

2) Introduction: "Meanwhile, non-kinetochore microtubules extend from opposite spindle poles" - Not all non-kinetochore microtubules extend from the poles. Many are formed within the spindle.

Authors: We simplified this sentence: "Meanwhile, non-kinetochore microtubules interact in an antiparallel fashion in the central part of the spindle, thus forming overlap regions."

3) In the concluding paragraphs authors discussed how stability of the certain parameters such as a length of antiparallel overlap can be conjugated with reduced by PRC1 knockdown interkinetochore tension (Page 12). They also discuss thinner bridging fibers after the knockdown procedure. However, the discussion is not directly linked to their own recently published model (Nature Communications 7, 2016). This model explicitly predicts that the forces at the pole and at the kinetochore depend on the bridging fiber thickness.

Authors: We have completely revised the concluding paragraphs, motivated by the reviewers' suggestions, and added the following discussion linking the new data with the model: "PRC1 knockdown resulted in thinner bridging fibers and reduced interkinetochore distance, but the spindle shape did not change significantly. According to our model [23], the compression in the bridging fiber counteracts the tension at the end of the k-fiber. Thus, when both forces are reduced, the spindle shape can remain unchanged, which is in agreement with our measurements."

4) Formation of PRC1 homodimer required for the effective interaction with kinesin-4 and microtubule binding affinity is believed to be triggered at the onset of anaphase. This should be considered in the discussion.

Authors: This issue is now considered in the discussion on page 12: "Previous studies have suggested that the formation of PRC1 homodimers, which is required for the interaction with kinesin-4 and for microtubule binding, is triggered at the onset of anaphase [10, 13, 15]. Yet, antiparallel overlaps containing PRC1 have also been reported in metaphase spindles [11, 23]..."

5) Fig 1. 3D projections in A and B are a bit confusing because the orientation of the spindle axis is not marked in the individual panels. Reading these panels requires a certain level of expertise in spindle architecture.

Authors: To help readers better understand the orientation of the spindle in 3D projections, 3D coordinate system represented as a cuboidal box is introduced in Fig. 1A, Fig. 1B, Expanded view Video 1 and Expanded view Video 2. We modified the caption of Fig. 1A: "Images of different z-slices (central plane of the spindle z=0, two images below, z=-4 µm and z=-2 µm, and above, z=+3 µm and z=+5 µm), maximum projection of a z-stack (max z), and 3D projections (3D) with the 3D coordinate system represented as a cuboidal box that indicates different spindle orientations are shown." We also added a similar sentence about the 3D box in Materials and methods and in Movie

captions (Expanded view Videos 1 and 2).

6) Fig 3C. The white line on the image obscures what exactly was contoured. In fact, it appears that both microtubules and PRC1 bundle arc upwards, not downwards as the schematic shows. It is important to show both the real image and the contour in parallel. Further, contour lines that connect spindle poles may be problematic if the centromere axis (which should be parallel to the PRC1 bundle) is significantly tilted with respect to the spindle axis. Clear examples of these tilted PRC1 bundles are present in Fig.1A. How was this issue dealt with?

Authors: We introduced new images with and without the contours in Fig. 3C and added the following sentence in the caption of Fig. 3C: "Images of the same spindle without (left) and with the tracked contour (right) are shown." We added the following explanation in the Image analysis section of Materials and methods: "In HeLa cells stably expressing tubulin-GFP that were immunostained for PRC1, we tracked a 5-pixel-thick pole-to-pole contour of tubulin-GFP signal of the sister k-fibers and the corresponding bridging fiber that spans between them. The positions of the spindle poles were estimated as the merging points of k-fibers. The bundles were tracked manually, point-by-point along the curved line, following the tubulin-GFP signal path (note that the bundles that disappeared in the z-direction were not tracked)." Thus, even when the sister kinetochore axis was tilted with respect to the spindle axis, the tracked contour covered the k-fibers and the bridging fiber.

7) Fig 3E. How exactly is the Lprc1 value determined? The scan is understandably noisy so the exact width of the peak cannot be set arbitrarily. What signal processing routines are applied?

Author: To address this question we explained in more detail the method of analysis in the section Image analysis of Materials and methods: "The length of the PRC1-labeled overlap region, LPRC1, was manually determined as the width of the peak of the PRC1-GFP signal intensity in the central part of the contour. The width of the peak was defined as the distance between the positions at the base of the PRC1-GFP peak where the PRC1-GFP signal intensity is roughly equal to the mean value of the PRC1-GFP signal intensity along the contour on either side of the peak."

8) Expanded view Video 3. The intensity of magenta signal increases abruptly between 143 and 156 seconds. It looks like the acquisition parameters were changed; however, such a change is not reported.

Authors: To clarify this issue, we added the following sentence in the Image acquisition section of Materials and methods: "In the cases when the transiently expressed mRFP-CENP-B significantly bleached during the experiment, the power of the white light laser (575 nm) was increased during the acquisition, which did not affect the measurements because the mRFP-CENP-B signal intensity was not quantified." We also added a note in the caption of Expanded view Video 3 (MovieEV3): "Note that the power of the white light laser (575 nm) was increased between 143 and 156 seconds."

9) Fig 4A. The signal/noise ratio is quite different in control vs. PRC1 siRNA. Does this imply that the total amount of tubulin incorporated into spindle (and individual k-fibers) is affected by PRC1 siRNA? Why is the spindle on the right so dim? The reason I bring up this issue is that if the number of microtubules within K-fibers and other bundles decreases upon PRC1 depletion, then the changes in inter-kinetochore distances could be a consequence of the lower number of microtubules, not a direct effect of PRC1 depletion. It may be important to reveal absolute values of Ib/Ibk not just the ratios.

Author: We thank the reviewer for pointing out this important issue. We added the absolute values of Ib, Ibk, and Ik = Ibk - Ib in Table 2, and the following text to Results, page 11:
"The signal intensity Ib was reduced by roughly 28% after PRC1 knockdown, which we interpret as the reduction in the number of microtubules in the bridging fiber (Table 2). On the contrary, the signal intensity Ik = Ibk - Ib, which corresponds to the number of microtubules in the k-fiber, was not affected significantly by PRC1 siRNA treatment (p=0.6, Table 2)."

Referee #2:

Polak et al. report on "bridging fibres" of the mitotic spindle. A previous publication from the Toli lab described these fibres, but now this paper proposes that there is a one-to-one association between bridging fibres and sister kinetochore pairs. It's an interesting piece of work and will be of interest to those of us working on spindle structure. Overall, I think the case is good. The three main observations here are:

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3. Colocalisation of PRC1-positive spots with kinetochores in live cell imaging experiments.

Two comments:

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Authors: We thank the reviewer for this important suggestion, which is similar to Point 1 by Referee 3. To improve this part, we added cross-correlation analysis on pages 7 and 8: "To quantify to which extent the kinetochores move in a correlated manner with different PRC1-labeled fibers in the spindle, we performed cross-correlation analysis [35] on the acquired trajectories. Our analysis revealed high correlation of movement between a kinetochore pair and the coupled PRC1-labeled fiber, with a median correlation coefficient of 0.93 (n=12). The correlation coefficient decreased with an increasing distance between the PRC1-labeled fiber and the kinetochore pair: the median correlation coefficient was 0.61 for the nearest neighbor fiber that was not coupled to the kinetochore pair, 0.35 for the next nearest neighbor, and 0.02 for a randomly chosen fiber (n=14-18 fibers in each group, Fig 2D; examples of trajectories are shown in Fig EV2C). These results indicate that kinetochores typically move together with their coupled PRC1-labeled fiber, whereas the correlation of movement with neighboring fibers decreases in a distance-dependent manner and vanishes for remote fibers."

2) Despite the correlations being good evidence for a one-to-one relationship, I somehow think it's a bit strong to say that ALL kinetochore pairs are associated with a bridging fibre. This statement leaves no wriggle room for i) unpairing and repairing dynamics between kinetochores and bridging fibres, ii) newly-attached or newly-syntelic kinetochores becoming paired. Both of these things probably happen and so the authors should think about whether it's wise to imply that the relationship is obligatory, i.e. one-to-one. After all, ~12% of PRC1 bundles are kinetochore free and ~6% of kinetochores have no PRC1 associated. There is experimental error, sure, but this is not one-to-one.

Authors: We agree with the reviewer. A similar issue was raised by Referee 1 (point 1). We changed throughout the paper the expressions "all" and "one-to-one" into "almost all" and "nearly all". We also added the following paragraph in the discussion: "The dynamics of live spindles revealed that PRC1-labeled fibers spend most of the time moving together with their associated kinetochores, with occasional uncoupling and recoupling events. Thus, the small fraction of PRC1-labeled fibers that were found not coupled with kinetochores in fixed cells likely represents bridging fibers that were in a dissociated state from their kinetochores at the time of cell fixation. Uncoupled fibers and kinetochores may be found more frequently in prophase, during the formation of the structure

comprising sister kinetochores, k-fibers, and their bridging fiber [36]."

Referee #3:

In their most recent paper about bridging microtubules, Polak et al perform a systematic analysis to understand the extent to which the overlap fibers are found in metaphase spindles, and to determine whether each kinetochore pair is indeed associated with such fibers, or whether only a subset of KT pairs are associated with such fibers. Their data indeed supports the notion that there is a one-to-one association between PRC1-labeled MT bundles and kinetochore pairs (as indicated by the title). The current work is thorough and carefully performed. I only have a few comments below addressing specific concerns, one of which raises the question of whether the proposed function of these fibers (to provide shape to the spindle) is supported by their work.

Besides those concerns outlined below, it's unclear whether the current body of work expands significantly on their previous work (their Nature Communications paper), other than to describe the presence of the overlap fibers throughout the spindle (rather than just at the edges). This may be due in part to the fact that throughout much of the manuscript, there is little explanation of the reasoning behind a particular experimental approach. Moreover, the discussion offers little other than a rehashing of the results. For instance, why did the authors look for a relationship between PRC1 signal and distance from the long axis of the spindle? Why did they explore the relationship between chromosome number and spindle length/width? Why did the authors bother to look at endogenous PRC1, rather than focus on episomal PRC1-GFP? What can be surmised from their findings (what does it mean that there is no relationship between PRC1 intensity and distance from spindle long axis?)? Other than describing the ubiquity of the overlap fibers throughout the spindle, I'm not sure what new findings are described in this current manuscript. I hope the comments below help the authors improve the clarity of their manuscript.

Major points:

1) As shown in Fig. 2, the majority of PRC1 labeled bundles indeed appear to move in a similar pattern to that of the associated kinetochore pair. However, could these correlated movements be due to the entire spindle moving as a cohesive unit? As a control, it would be helpful to compare the movement of non-closely associated, randomly chosen PRC bundles and kinetochore pairs.

Authors: We thank the reviewer for this suggestion, which is similar to Point 1 by Referee 2. To compare the movement of non-closely associated, randomly chosen PRC1 bundles and kinetochore pairs, we added the following analysis on pages 7 and 8: "To quantify to which extent the kinetochores move in a correlated manner with different PRC1-labeled fibers in the spindle, we performed cross-correlation analysis [35] on the acquired trajectories. Our analysis revealed high correlation of movement between a kinetochore pair and the coupled PRC1-labeled fiber, with a median correlation coefficient of 0.93 (n=12). The correlation coefficient decreased with an increasing distance between the PRC1-labeled fiber and the kinetochore pair: the median correlation coefficient was 0.61 for the nearest neighbor fiber that was not coupled to the kinetochore pair, 0.35 for the next nearest neighbor, and 0.02 for a randomly chosen fiber (n=14-18 fibers in each group, Fig 2D; examples of trajectories are shown in Fig EV2C). These results indicate that kinetochores typically move together with their coupled PRC1-labeled fiber, whereas the correlation of movement with neighboring fibers decreases in a distance-dependent manner and vanishes for remote fibers."

To address the possibility of the entire spindle moving as a cohesive unit, we added the following sentence to Materials and Methods: "To avoid the possible effect of trajectories being the result of the entire spindle moving as a cohesive unit, we calculated the trajectories of kinetochore pairs and of PRC1-labeled bundles with respect to the spindleís center of mass in each image." We also added "with respect to the spindleís center of the mass" to the caption of Fig. 2.

2) If reducing PRC1 levels by siRNA reduces bridging MT number, and the spindle shape is presumably reliant on these bridge fibers (akin to the wooden rod and rope analogy described in Iva's recent Cell Cycle commentary), then why does the shape of the spindle not change upon PRC1 knock-down (at least as measured from spindle height and width in Table 2)? Maybe I'm missing something, but this seems to be a key feature of bridging MTs: that they provide sufficient rigidity

to support the curved shape of the spindle. The authors alluded to something similar in their Nature Communications paper (cells with thicker fibers have a curved shape, while those with thinner fibers have a diamond shape).

Authors: We thank the reviewer for raising this interesting point, which is now discussed in the concluding paragraphs: "PRC1 knockdown resulted in thinner bridging fibers and reduced interkinetochore distance, but the spindle shape did not change significantly. According to our model [23], the compression in the bridging fiber counteracts the tension at the end of the k-fiber. Thus, when both forces are reduced, the spindle shape can remain unchanged, which is in agreement with our measurements."

3) The authors note a reduction in inter-KT distance upon PRC1 knock-down, and attribute this to a reduction in bridging fiber thickness (i.e., # of MTs making up a fiber). It's unclear to me how reduction in bridging fiber thickness results in reduced inter-KT distance. Given the authors have previously generated a theoretical model of spindles, it would be helpful if they either applied the model to help explain this observation, or discussed data previously acquired to help the reader understand where the force imbalance is (i.e., what is the source) that leads to the reduced inter-KT distances.

Authors: We thank the reviewer for commenting on this problem. We removed the statements that the observed reduction in interkinetochore distance upon PRC1 knockdown results from the reduction in bridging fiber thickness, because this conclusion cannot be drawn from the data presented in this paper. We do not use our published model to explore this issue because our model does not have a mechanism of tension generation. In a separate study, we are currently extending our model to include motor proteins, which will allow us to provide predictions for the change in interkinetochore tension as a function of the bridging fiber characteristics.

Minor points:

1) It is unclear why the authors tested for a correlation between PRC1 signal (or inter-KT distances), and distance from the spindle long axis? Perhaps I'm missing something, but I don't understand why there would be any relationship between these two parameters. This would imply that the further 'up' or 'down' laterally one considers from the long axis (with respect to a bird's eye view of the spindle), there might be a change in the density of PRC1. I understand that, according to EV3, there is no relationship, but isn't this expected? Unless the authors were expecting a relationship between degree of kMT curvature and bridge MT number? In any case, this point needs to be clarified.

Authors: We agree with the reviewer and we clarified our reasoning by adding the following text on page 10: "K-fibers together with the associated bridging fibers at the periphery of the spindle are longer and more curved than those near the spindle long axis. Thus, we asked whether PRC1 signal parameters in the bridging fiber depend on the distance of the fiber from the spindle long axis." and at the end of the same paragraph: "These results suggest that all bridging fibers in the spindle have a similar length of the PRC1-bound antiparallel overlap zone and a similar amount of PRC1, regardless of the length and curvature of the associated k-fibers."

2) The following sentence is confusing as worded: "Next, we tested whether the distance between sister kinetochores and spindle proportions is affected by PRC1 siRNA." It sounds like they are testing for distances between 'spindle proportions' as well between sister KTs. The sentence should be reworded.

Authors: We modified the sentence: "Next, we tested whether PRC1 siRNA affected the interkinetochore distance and spindle length and width."

3) Figure 4E appears to be monochromatic in spite of the label indicating the presence of green (tubulin) and magenta (PRC1). The legend indicates "Only AlexaFluor555 channel is shown," in which case the label above the figure needs to be changed.

Authors: We removed the unnecessary label. The legend now indicates only PRC1-AleaFluor555 (magenta).

4) Similarly, Fig. 4G indicates the presence of green and magenta, but I only see magenta.

Authors: The legend now indicates only PRC1-AleaFluor555 (magenta).

5) Also, the plot in Fig. 4F has tubulin-GFP and PRC1-AlexaFluor555 labels above it. Unless these labels are meant for Fig. 4E? The labeling is quite confusing throughout much of Fig. 4 (same confusion in panel H).

Authors: We removed the unnecessary labels in Fig. 4F and Fig. 4H.

6) In contrast, the two-color images in Fig. 2D are unlabeled. I realize the legend indicates what the colors represent, but it would be easier for the reader to see the labels on the figure.

Authors: We wrote the legend for images in Fig. 2E (previous Fig. 2D).

7) The text points to Fig. 4G twice in a row on page 11 ("PRC1-GFP were altered by PRC1 knockdown (Fig 4G, Fig 4G"...)

Authors: Corrected: "(Fig 4G, Fig EV4E, Fig EV4F, Table 2)."

8) It is unclear how PRC1 "density" was determined, or what that even refers to (Fig. 1G). Are they referring to the size of the fluorescent spot (the units suggest as much)? Or, are they referring to the # of PRC1 spots per unit area? This needs to be clarified, and the text needs to include an explanation of the relevance of this data.

Authors: We rewrote this paragraph in a clearer manner: "To examine the spatial distribution of bridging fibers in spindles with different numbers of chromosomes, we measured the density of PRC1-labeled fibers coupled with kinetochores, i.e., their number per unit area in the equatorial plane of horizontal and vertical spindles. We found that the density does not depend significantly on the number of coupled pairs (Fig 1G, Fig EV1F). Thus, by accommodating its width the spindle maintains the neighboring bridging fibers at similar distances regardless of the total number of chromosomes in the spindle."

2nd Editorial Decision 17 November 2016

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the three referee reports that you will find enclosed below. As you will see, all three referees now support the publication of your manuscript in EMBO reports. Before we can proceed to formal acceptance, I have some editorial requests to be addressed in a final revised version.

- Would it be possible to have a higher quality image of the Western blots in the Figures EV3A and EV4A (more equal exposure, markers are very dark, other areas very bright).

- It seems that some panels in Figure EV1 appear also in Figure 1. Could you please indicate and explain that better in the text and/or the figure legends.

REFEREE REPORTS

Referee #1: The authors have addressed all concerns raised during the evaluation of the original submission. The revised manuscript is significantly improved and I recommend it be published.

Referee #2: I'm happy with the revisions the authors have made in response to my comments.

Referee #3: Polak et al. have done a very nice job addressing all of my comments and concerns. I think the cross correlation analysis in particular (Fig. 2D) provides very convincing evidence that the PRC1 fibers are indeed associated with kinetochore pairs. All in all, I think their work will make a nice contribution to the field.

2nd Revision - authors' response 22 November 2016

Please find attached our revised manuscript “One-to-one association between PRC1-labeled microtubule bundles and kinetochore pairs in metaphase.” We uploaded the files following your requests. The manuscript is formatted according to your instructions. For the Western blots, unfortunately, we do not have higher quality images of the Western blots in the Figures EV3A and EV4A. The current images in those Figures are the best that we could obtain with adjusted exposures. For Figure 1A and 1B and Figure EV1A and EV1B, the panels show the same spindle with more z-slices shown in Figure EV1. This is now indicated in both figure captions.
For publication of the original source data, we provide data points of selected graphs in an Excel file and the original Western blots as PDF files.

3rd Editorial Decision 25 November 2016

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Iva M. Tolić
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2016-43015V1

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

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B- Statistics and general methods

Please fill out these boxes (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	For main experiment sample size was chosen to ensure more than 1000 investigated kinetochores and PRC1-signals. For other experiments and controls number of cells was chosen to be roughly 10.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Inclusion criteria was: proper spindle orientation and bipolarity, metaphase plate alignment, presence signal intensities of fluorescently tagged proteins.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	From a population of cells in the flask, 1000000 cells were randomly chosen for treatment.
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Based on a previous study kinetochore pair and PRC1-labeled bundle were defined as associated if the distance between them was smaller than 0.3 µm.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Student's t-test is statistical data analysis appropriate for this research. It was used to determine if groups of data are significantly different from each other.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Normal distribution of the data was confirmed by the sampling distribution of the mean that is normal for cells with ≤70 and those with >70 chromosomes tested with two approaches, spindles with their long axis oriented roughly parallel to the imaging plane (horizontal spindles), spindles with their long axis oriented roughly perpendicular to the imaging plane (vertical spindles).
Is there an estimate of variation within each group of data?	For cells with ≤70 and those with >70 chromosomes, standard deviation was calculated within each group of data by calculating separate measures for each group.
Is the variance similar between the groups that are being statistically compared?	Varioation was calculated between groups using three different approaches, spindles with their long axis oriented roughly parallel to the imaging plane (horizontal spindles), spindles with their long axis oriented roughly perpendicular to the imaging plane (vertical spindles) and by using different imaging settings to image only the central planes of horizontal spindles.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	PRC1 Antibody (H-70): sc-8356 (http://1degreebio.org/reagents/product/1407067/?qid=1324048)
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	HeLa-TDS cells were permanently transfected and stabilized (courtesy of Mariola Chacon) using pEGFP-α-tubulin plasmid, which was acquired from Frank Bradke (Max Planck Institute of Neurobiology, Martinsried). HeLa-Kyoto BAC lines stably expressing PRC1-GFP are courtesy of Ina Poser and Tony Hyman (MPI-CBG, Dresden). Cells were negative for mycoplasmas.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA

10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	NA
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA