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RanGTP induces an effector gradient of XCTK2 and importin α/β for spindle microtubule cross-linking

Stephanie Ems-McClung, Mackenzie Emch, Stephanie Zhang, Serena Mahnoor, Lesley Weaver, and Claire Walczak

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July 22, 2019

Re: JCB manuscript #201906045

Dr. Claire E Walczak
Indiana School of Medicine - Bloomington
Medical Sciences
915 E. 3rd St. MY 262
Bloomington, IN 47405

Dear Claire,

Thank you for submitting your manuscript entitled "RanGTP induces an effector gradient of XCTK2 and importin α/β for spindle microtubule cross-linking". We apologize for the delay in providing you with a decision.
In any case, the manuscript was assessed by two expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

You will see that both reviewers are supportive of the study but have raised a few concerns that will need to be addressed before the paper will be suitable for publication in JCB. Reviewer #2 has raised only minor concerns which should be addressable with changes to the text. Reviewer #1, however, has raised several more substantive concerns. After assessing this reviewer's comments carefully, we agree that further corroboration of FLIM data will be necessary in the revised manuscript (reviewer #1, point #1). However, although we agree that providing further insight into the nature of the mechanism by which importin α/β binding inhibits antiparallel sliding by XCTK2 (reviewer #1, point #3) would increase the impact of the study, given that this is a Report, we feel that such an extension is beyond the scope of the current paper. Thus, you will not need to address this point with new experiments. In addition, we feel that the issue regarding MT polarity (reviewer #1, point #2) is likely due to confusion arising from the similar length of plus- and minus-ends segments in the cartoons shown in Figure 3, panel A. In reality (i.e. the images in panel B), minus-end segments are significantly shorter than the plus-end ones and thus, the polarity claims appear to be valid. Therefore, we ask that you do your best to make the figure clearer and better describe how segment-labeled microtubules were prepared - we do not feel, however, that you will need to repeat these experiments as the reviewer suggests.

Please be sure to provide responses to each of the reviewers' comments in your rebuttal document, even if you will not be addressing the points experimentally.

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

GENERAL GUIDELINES:
Text limits: Character count for a Report is < 20,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

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IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.

Supplemental information: There are strict limits on the allowable amount of supplemental data. Reports may have up to 3 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

Our typical timeframe for revisions is three months; if submitted within this timeframe, novelty will not be reassessed at the final decision. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

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

Sincerely,

Alexey Khodjakov, PhD
Monitoring Editor
JCB

Tim Spencer, PhD
Deputy Editor
Journal of Cell Biology

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

The RanGTP gradient is typically thought of in terms of localized release and activation of spindle assembly factors (SAFs) that promote nucleation, stabilization and organization of MTs to promote bipolar spindle assembly. This study puts an interesting "spin" on this notion by pointing out that the presence of a RanGTP gradient actually produces multiple effector gradients within the spindle: 1) the chromatin-centered RanGTP gradient that liberates SAFs from import receptors, and 2) polar gradients of cargoes bound to import receptors such as importin a/b. In this case the authors argue

that these distinct spatial gradients regulate the function of the minus-end directed motor XCTK2, which the Walczak group previously demonstrated is a target of the Ran pathway. The authors propose that the conventional chromatin-centered RanGTP gradient liberates XCTK2 from importin a/b around DNA where the motor can bind MT and preferentially crosslink and slide anti-parallel MTs. However, in the polar regions where RanGTP concentration is lower, Impa/b binding to XCTK2 promotes parallel MT crosslinking and sliding via inhibition of its anti-parallel binding capacity.

Typical RanGTP gradient models envision that release of cargo from Imp a/b locally activates SAFs around DNA, this work proposes a variation on this classical model in which Impa/b binding to cargoes should not be viewed as so black and white. In the case of XCTK2 they argue that a/b binding near poles biases motor activity towards crosslinking and sliding parallel MTs to promote spindle pole focusing and centrosome clustering. I appreciate that this would be an interesting addition to the classical view on spatial regulation by the RanGTP gradient, but I have concerns about the presented data supporting the major conclusion. Most notably, I did not find the MT sliding data as presented to be convincing. This and other concerns outlined below should be addressed before the work is further considered for publication in JCB.

Major concerns:
1) The in vitro data with purified components in figure 1 nicely demonstrate that there is FRET between the XCTK2 and Imp a, however, I found the FLIM data in the egg extracts to be less convincing. The representative lifetime image for the YXCTK2 should look like the inverse of the RanGTP gradient but this is not that evident (based on 2E there is only a ~3% change in lifetimes between the chromatin region and the poles). Given the importance of convincingly demonstrating this spatial interaction gradient, I feel further validation of the FLIM results in the extract is warranted. What happens if increasing concentrations of RanQ69L are added to these reactions? It is shown in Figure 4 that increasing amounts of XCTK2 bind the spindle with increasing RanQ69L additions. Does Impa-CyPet (+YXCTK2) exhibit increased lifetimes (decreased FRET) under conditions of increasing RanQ69L concentration? The addition of this data would strengthen confidence in the FLIM measurements in extract.
2) The regulation of XCTK2 crosslinking and sliding activities by Importin a/b is another centerpiece of this study. However, in looking at the representative images in Figure 3B and the supplemental videos, it is unclear how the authors (or the reader) differentiate between parallel and anti-parallel sliding events. Since the authors used striped/segmented MTs in their assays it was not obvious how the plus and minus ends were determined. While the quantifications in Figure 3C-I are thorough, I don't really know how to evaluate them since it is unclear to me how the polarity was defined with confidence. Given the critical importance of these data to their functional conclusion, it is important that these experiments be repeated with clear polarity marked MTs.
3) If it is true that importin a/b binding inhibits antiparallel sliding by XCTK2 but not its parallel sliding activity, what is the mechanism by which this regulation is achieved? It would improve this work if there was investigation of the hypothesis that there are two separable MT binding domains in the tail that are differentially sensitive to inhibition by imp a/b. Since the basic nature of an NLS makes it a possible MT binding motif perhaps the NLS itself is the MT binding motif that favors anti-parallel binding and sliding. Using better polarity marked MTs, what are the binding and sliding characteristics of the YXNLS protein? It would be very interesting if it was preferential for parallel MTs. Regardless of the outcome, this protein would also serve as a necessary control for the experiments presented in Figure 3 with YXCTK2 since the addition of Imp a/b should not affect its activity in the assay.

Minor concerns:

1) The YXCTK2 and YXNLS nomenclature made it difficult to keep some of the experiments straight. While it saves space, it would be helpful to the reader if the proteins were labeled more unambiguously (e.g. YPet-XCTK2 and YPet-XCTK2-deltaNLS).
2) The idea that this mechanism is important in clustering multiple centrosomes in cancer cells is speculative and not directly investigated in this study. I think it's up to the authors as to whether they keep it as a discussion point, but I don't think it warrants being stated in the abstract especially since it gave me the impression that centrosome clustering would be investigated in the manuscript.
3) The data in Figure S1B showing that the presence of ImpB increases the FRET between YXCTK2 and Impa-CyPet could be added to Figure 1.
4) Figure legends for supplemental videos state that the plus-ends of the cargo MTs are labeled with an asterisk but I did not see an asterisk in the videos.
5) The model in Figure 5A and B is presented such that, in the polar regions, only some XCTK2 motors ("anti-parallel") are bound to Imp a/b while the "parallel" motors are not. I don't view this as an accurate representation. This gives the perception that imp a/b somehow differentiates between parallel and anti-parallel motors. I think it is more reasonable to present the model in a manner that better reflects the way the authors are thinking about the regulatory mechanism (based on the text). Specifically, Imp a/b should bind most or all the XCTK2 molecules near the poles but this binding only inhibits anti-parallel sliding activity by XCTK2 but not its parallel sliding activity.

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

REVISED REVIEW
Using a combination of live-microscopy tools, Walczak and colleagues provide evidence for how the Ran pathway promotes selective cross-linking of parallel microtubules at the spindle poles to cluster centrosomes. Ran, required for spindle assembly, forms a gradient around chromatin; how it controls events close to spindle poles where the gradient would dissipate is not fully understood. One hypothesis has been that the Ran gradient that dissipates towards the poles will influence a distinct set of spindle associated factors. In support of this hypothesis, the authors show that the localization and activity of Xenopus Kinesin-14, XCTK2, in the spindle is spatially controlled by Ran which modulates XCTK2 binding affinity to microtubules. They find that importins could reduce the microtubule affinity of the XCTK2 tail domain through competition. Although kinesin-14 can bundle both parallel and anti-parallel microtubules, importins preferentially inhibit XCTK2-mediated anti-parallel MT cross-linking and sliding activity. Thus, the study highlights how the Ran pathway promotes Kinesin-14 mediated parallel microtubule cross-linking at the spindle poles to cluster centrosomes. The findings reported here can significantly impact our understanding of mechanisms underpinning centrosome clustering frequently observed in human cancer cells. The authors propose that cancer cells with excess chromosomes could set up a steep Ran gradient and bias the clustering of centrosomes as it is essential for cell survival. With drugs targeting HSET (human Kinesin-14) being considered in the market, this paper provides excellent insight into how the Ran pathway may promote the survival and rescue of cancer cells, if the authors could query this in cell lines.

Overall the data is clearly presented and text well written. The methods are outlined in detail with appropriate statistical analysis. I am satisfied with the data interpretations and conclusions. I think the authors have also made a measured but important proposal at the very end of the manuscript. This could be bolstered further through a minor experiment, if they would like to highlight in the

summary section as well (See below).

I have no major queries, listed below are minor queries.

Minor queries
1. How large is the tail region that bears the NLS (YXtail)? Is the NLS always exposed in the MT-bound and unbound forms of the molecule? Will this have an impact on their model?
2. For Fig 2A, would it be useful/valuable to present FLIM images from multiple Z-sections? This could help strengthening the FLIM data - this is an important part of the study and could be demonstrated a bit more clearly.
3. Does Rango-2 expression alter the steepness of Ran gradient? Are there controls to infer Rango-2 induced differences across the dataset?
4. The proposed impact on cancer cells could be highlighted in the summary section - this is a clinically impactful idea that emerges from this basic biology work. It could potentially be easily tested by exogenously overexpressing XCTK2 mutant (for example the tail domain mutant) in a cell line exhibiting centrosome clustering defect. This line of experiment(s) is/are not critical for the current manuscript but will help bolster their proposal on centrosome clustering in cancer cells.
5. Figure 5B is somewhat unclear - Figures 5A and 5C make clear points and so Figure 5B could be omitted or presented in the context of the spindle.

Dear Alexey,

Re: JCB manuscript #201906045

Please find our revised manuscript "RanGTP induces an effector gradient of XCTK2 and importin α/β for spindle microtubule cross-linking". Included here are the point-by-point responses to both the editorial and reviewers' comments. We are thankful for the helpful suggestions and feel we have addressed the concerns with the inclusion of new data as well as revisions to both the text and figures. Together these changes have resulted in an improved manuscript that more clearly and definitely demonstrates our key points. We hope you and the reviewers will find the changes satisfactory and look forward to hearing from you.

Response to the Reviews

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

The RanGTP gradient is typically thought of in terms of localized release and activation of spindle assembly factors (SAFs) that promote nucleation, stabilization and organization of MTs to promote bipolar spindle assembly. This study puts an interesting "spin" on this notion by pointing out that the presence of a RanGTP gradient actually produces multiple effector gradients within the spindle: 1) the chromatin-centered RanGTP gradient that liberates SAFs from import receptors, and 2) polar gradients of cargoes bound to import receptors such as importin a/b. In this case the authors argue that these distinct spatial gradients regulate the function of the minus-end directed motor XCTK2, which the Walczak group previously demonstrated is a target of the Ran pathway. The authors propose that the conventional chromatin-centered RanGTP gradient liberates XCTK2 from importin a/b around DNA where the motor can bind MT and preferentially crosslink and slide anti-parallel MTs. However, in the polar regions where RanGTP concentration is lower, Impa/b binding to XCTK2 promotes parallel MT crosslinking and sliding via inhibition of its anti-parallel binding capacity.

Typical RanGTP gradient models envision that release of cargo from Imp a/b locally activates SAFs around DNA, this work proposes a variation on this classical model in which Impa/b binding to cargoes should not be viewed as so black and white. In the case of XCTK2 they argue that a/b binding near poles biases motor activity towards crosslinking and sliding parallel MTs to promote spindle pole focusing and centrosome clustering. I appreciate that this would be an interesting addition to the classical view on spatial regulation by the RanGTP gradient, but I have concerns about the presented data supporting the major conclusion. Most notably, I did not find the MT sliding data as presented to be convincing. This and other concerns outlined below should be addressed before the work is further considered for publication in JCB.

Major concerns:
1) The in vitro data with purified components in figure 1 nicely demonstrate that there is FRET between the XCTK2 and Imp a, however, I found the FLIM data in the egg extracts to be less convincing. The representative lifetime image for the YXCTK2 should look like the inverse of the RanGTP gradient but this is not that evident (based on 2E there is only a ~3% change in lifetimes between the chromatin region and the poles). Given the importance of convincingly demonstrating this spatial interaction gradient, I feel further validation of the FLIM results in the extract is warranted. What happens if increasing concentrations of RanQ69L are added to these reactions? It is shown in Figure 4 that increasing amounts of XCTK2 bind the spindle with increasing RanQ69L additions. Does Impa-CyPet

(+YXCTK2) exhibit increased lifetimes (decreased FRET) under conditions of increasing RanQ69L concentration? The addition of this data would strengthen confidence in the FLIM measurements in extract.

Thank you for the insightful suggestion of adding Ran to the FLIM experiment. We have done the experiment and include the new data in Figure 5 of the revised manuscript. In addition, we have edited the text of the manuscript to clarify a few points of confusion regarding the interpretation of the data. Below is a point by point response to the concerns.

1. The RanGTP gradient in our control spindles shown in Fig. 4 B has a ~3% difference in lifetime from the chromatin to the poles. This difference is similar to the ~4% difference found in the initial paper that described the RanGTP gradient biosensor in cells (Kaláb, Nature 2006), showing that we are recapitulating the detection of the gradient similarly to what has been reported previously. The addition of 10-20 uM Ran increases the steepness of the RanGTP gradient in our extract spindles to a ~5% difference between chromatin and poles. Thus, the ~3% change we see with YPet-XCTK2 and importin alpha-CyPet in Fig. 2 C would also be consistent with the existence of a gradient with differences in the range as what has been shown for the RanGTP gradient. It is important to note that the differences in lifetime between the chromatin and poles for YPet-XCTK2 are also significantly different from zero (one sample t test, $p < 0.001$), which is consistent with the presence of a gradient. In addition, the lifetimes of YPet-XCTK2 across the spindle are significantly different from importin alpha-CyPet alone as well as from YPet-XCTK2-ΔNLS, which can bind to microtubules in the spindle but cannot interact with importin alpha, demonstrating that it is insensitive to the RanGTP gradient in the spindle. We feel that these findings are consistent with the ability of our XCTK2 biosensor to be sensitive enough to show a gradient of association.

2. As predicted by the reviewer, addition of Ran to extracts followed by FLIM of spindles with importin alpha-CyPet and YPet-XCTK2 resulted in decreased FRET and longer lifetimes, which is now shown in Fig. 5, A-C. While we saw decreased association of importin alpha-CyPet and YPet-XCTK2 across the spindle, we did not see a change in the steepness of the association. This result is consistent with our favored model in that it's not the steepness of the RanGTP gradient, but rather the presence of the gradient that sets up an effector gradient of importin alpha/beta association with XCTK2. The steepness of the effector gradient may subsequently be regulated by the local concentrations XCTK2 as well as its affinity of interaction with importin alpha/beta. Thus, differential affinities of SAFs for importin alpha/beta would act as the rheostat for the effector gradient rather than the RanGTP gradient per se. Second, while the enhanced RanGTP gradient shifted the distribution of YPet-XCTK2 on the spindle, the distribution may not have been shifted enough to be able to detect changes in the steepness of the effector gradient because YPet-XCTK2 levels were already saturated on the spindle. Alternatively, due to the high affinity of XCTK2 and importin alpha/beta, it may not be possible to enhance the RanGTP gradient enough to alter this interaction in the spindle. Due to the limitations in sensitivity of FLIM in extracts we could not try lower concentrations of YPet-XCTK2 nor do dilution series of added Ran to test these possibilities.

3. The use of the word 'inverse' was probably not the best choice and likely led to the concept that the XCTK2 effector gradient would be opposite of and equivalent to the RanGTP gradient. While the differences in lifetime measured for the RanGTP and YPet-XCTK2 gradients are similar, we shouldn't infer that they are equivalent as they use different biosensors for detection of the

gradients. We have updated the text by using the words 'opposite' or 'opposing' to indicate that the XCTK2 effector gradient is high at the poles and low at the chromatin, which is the opposite direction of the RanGTP gradient that is high at the chromatin and low at the poles.

2) The regulation of XCTK2 crosslinking and sliding activities by Importin a/b is another centerpiece of this study. However, in looking at the representative images in Figure 3B and the supplemental videos, it is unclear how the authors (or the reader) differentiate between parallel and anti-parallel sliding events. Since the authors used striped/segmented MTs in their assays it was not obvious how the plus and minus ends were determined. While the quantifications in Figure 3C-I are thorough, I don't really know how to evaluate them since it is unclear to me how the polarity was defined with confidence. Given the critical importance of these data to their functional conclusion, it is important that these experiments be repeated with clear polarity marked MTs.

We have lengthened the plus-ends of the MTs in the schematic in Fig. 3 A to provide more clarity. We also remade and annotated the videos to include the individual magenta and green channels so that readers can more clearly differentiate the polarity of the template and cargo MTs to see how the orientation of the cross-links were scored. In addition, we added additional details to the methods section on how we determined the polarity and orientation of the microtubules.

3) If it is true that importin a/b binding inhibits antiparallel sliding by XCTK2 but not its parallel sliding activity, what is the mechanism by which this regulation is achieved? It would improve this work if there was investigation of the hypothesis that there are two separable MT binding domains in the tail that are differentially sensitive to inhibition by imp a/b. Since the basic nature of an NLS makes it a possible MT binding motif perhaps the NLS itself is the MT binding motif that favors anti-parallel binding and sliding. Using better polarity marked MTs, what are the binding and sliding characteristics of the YXNLS protein? It would be very interesting if it was preferential for parallel MTs. Regardless of the outcome, this protein would also serve as a necessary control for the experiments presented in Figure 3 with YXCTK2 since the addition of Imp a/b should not affect its activity in the assay.

We too are very curious about how the tail can recognize two different orientations of MT cross-links. We have some interesting preliminary data to this end, but clearly establishing the existence of two MT binding domains is not straightforward. Our favorite model is that there are two MT binding sites in the tail: one for parallel cross-linking and one for anti-parallel cross-linking. We are currently trying to fine tune the identification of the MT binding regions. This will then provide a unique opportunity to carry out structural studies (Cryo-EM) to identify the different regions of interaction along with functional experiments for spindle assembly (depletion/addback) so that we can dissect how structure/function of the different cross-linking activities modulate spindle organization. We thank the editor for appreciating that such an amount of work is beyond the scope of the current paper, especially since it is a Report.

Minor concerns:
1) The YXCTK2 and YXNLS nomenclature made it difficult to keep some of the experiments straight. While it saves space, it would be helpful to the reader if the proteins were labeled more unambiguously (e.g. YPet-XCTK2 and YPet-XCTK2-deltaNLS).

We have changed the names of the proteins in the main body of the text so that it is easier for the reader to differentiate between the proteins. For the labeling of figures, we defined YPet-XCTK2 as YXCTK2 and YPet-XCTK2-ΔNLS as YXΔNLS for brevity.

2) The idea that this mechanism is important in clustering multiple centrosomes in cancer cells is speculative and not directly investigated in this study. I think it's up to the authors as to whether they keep it as a discussion point, but I don't think it warrants being stated in the abstract especially since it gave me the impression that centrosome clustering would be investigated in the manuscript.

We have taken this point out of the abstract but have left it in the text as an important and novel discussion point especially in light of the experiment requested by Reviewer #1 in major concern #1 that is now part of Figure 5.

3) The data in Figure S1B showing that the presence of ImpB increases the FRET between YXCTK2 and Impa-CyPet could be added to Figure 1.

Done!

4) Figure legends for supplemental videos state that the plus-ends of the cargo MTs are labeled with an asterisk but I did not see an asterisk in the videos.

Thank you for pointing this out as it was an oversight on our part, our apologies. We have fixed the annotation of the movies.

5) The model in Figure 5A and B is presented such that, in the polar regions, only some XCTK2 motors ("anti-parallel") are bound to Imp a/b while the "parallel" motors are not. I don't view this as an accurate representation. This gives the perception that imp a/b somehow differentiates between parallel and anti-parallel motors. I think it is more reasonable to present the model in a manner that better reflects the way the authors are thinking about the regulatory mechanism (based on the text). Specifically, Imp a/b should bind most or all the XCTK2 molecules near the poles, but this binding only inhibits anti-parallel sliding activity by XCTK2 but not its parallel sliding activity.

Thank you for highlighting some confusing issues with how we present our model in the text and the model figure. We have now made changes to the text and figure to help alleviate any confusion. Our idea is that importin alpha/beta can differentiate between parallel and anti-parallel MT cross-links through simple competitive inhibition. We hypothesize that as XCTK2 moves toward the spindle poles, it is influenced by the differences in the RanGTP gradient. Near the spindle poles, where RanGTP is low, importin alpha/beta bind to the XCTK2 tail and competitively inhibit anti-parallel MT cross-links but not parallel MT cross-links, which would help focus MT minus ends at the spindle poles. Alternatively, importin alpha/beta binding to the tail could prevent non-productive MT binding in the anti-parallel orientation, which would facilitate productive MT binding in the parallel orientation. In contrast, at the chromatin where importin alpha/beta binding is reduced, XCTK2 can cross-link MTs in both parallel and anti-parallel orientations. From our experiments at this time, we cannot deduce what portion of XCTK2 is bound by importin a/b at the poles. From our earlier work (Weaver et al., 2015), the turnover of XCTK2 at the poles is ~20 sec, which will allow for rapid turnover of interactions. In addition, our biochemical analysis shows that the anti-parallel sliding and MT tail binding by XCTK2 in the presence importin alpha/beta is reduced by ~50%, suggesting that the other half of the tail proteins are bound to MTs in the parallel orientation. In addition, XCTK2 can cross-link both parallel and anti-parallel MTs to similar extents, suggesting that if there are two MT binding domains in the tail, the affinities of the two MT binding domains may be similar. Thus, we would predict that at best ~50% of the XCTK2 would be bound by importin alpha/beta at spindle poles. For simplicity in the model in Fig. 5 A, we show only one

bound parallel motor and one unbound anti-parallel motor since we do not know the proportion of XCTK2 bound by importin alpha/beta.

We have also entertained the idea that importin alpha/beta binding to one site on the tail may co-exist with the tail binding to MTs through the other site, but we currently do not have any definitive evidence that a tail domain bound by importin alpha/beta can still bind MTs. Our MT binding data, where we see partial MT binding of the tail in the presence of importin alpha/beta, is also consistent with the idea that the portion of the tail that is bound to MTs is through a distinct MT binding site from the one inhibited by importin alpha/beta. Granted, the affinity of importin alpha/beta for XCTK2 appears tighter than the affinity of the tail to MTs in our assays, but these two assays were done with different concentrations of XCTK2/tail proteins and buffer conditions, making it difficult to compare in this way. We anticipate with our future endeavors into characterizing the tail as having one or two MT binding domains that we will be able to determine whether the tail can or cannot simultaneously bind importin alpha/beta and MTs.

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

REVISED REVIEW
Using a combination of live-microscopy tools, Walczak and colleagues provide evidence for how the Ran pathway promotes selective cross-linking of parallel microtubules at the spindle poles to cluster centrosomes. Ran, required for spindle assembly, forms a gradient around chromatin; how it controls events close to spindle poles where the gradient would dissipate is not fully understood. One hypothesis has been that the Ran gradient that dissipates towards the poles will influence a distinct set of spindle associated factors. In support of this hypothesis, the authors show that the localization and activity of Xenopus Kinesin-14, XCTK2, in the spindle is spatially controlled by Ran which modulates XCTK2 binding affinity to microtubules. They find that importins could reduce the microtubule affinity of the XCTK2 tail domain through competition. Although kinesin-14 can bundle both parallel and anti-parallel microtubules, importins preferentially inhibit XCTK2-mediated anti-parallel MT cross-linking and sliding activity. Thus, the study highlights how the Ran pathway promotes Kinesin-14 mediated parallel microtubule cross-linking at the spindle poles to cluster centrosomes. The findings reported here can significantly impact our understanding of mechanisms underpinning centrosome clustering frequently observed in human cancer cells. The authors propose that cancer cells with excess chromosomes could set up a steep Ran gradient and bias the clustering of centrosomes as it is essential for cell survival. With drugs targeting HSET (human Kinesin-14) being considered in the market, this paper provides excellent insight into how the Ran pathway may promote the survival and rescue of cancer cells, if the authors could query this in cell lines.

Overall the data is clearly presented and text well written. The methods are outlined in detail with appropriate statistical analysis. I am satisfied with the data interpretations and conclusions. I think the authors have also made a measured but important proposal at the very end of the manuscript. This could be bolstered further through a minor experiment, if they would like to highlight in the summary section as well (See below).

I have no major queries, listed below are minor queries.

Minor queries
1. How large is the tail region that bears the NLS (YXtail)? Is the NLS always exposed in the MT-bound and unbound forms of the molecule? Will this have an impact on their model?

The tail is 120 amino acids with a predicted MW of 13KDa. We would love to know whether the NLS is exposed or not in the bound and unbound forms, but there is not a crystal structure of this domain for any Kinesin-14. Secondary structure prediction indicates very little secondary structure, and the tail is likely disordered until it contacts a MT. We have included the size of the tail in the methods section and again when we discuss the implications for such a small domain to be able to recognize two different MT orientations.

2. For Fig 2A, would it be useful/valuable to present FLIM images from multiple Z-sections? This could help strengthening the FLIM data - this is an important part of the study and could be demonstrated a bit more clearly.

We agree that taking Z-sections could enhance the FLIM images by averaging multiple planes, but these FLIM images require 1-2 min to acquire a single focal plane, and in this time frame the spindle is apt to move and change shape, so this precludes the ability to take serial Z sections. Also keep in mind that the Xenopus spindles are gently "squashed" between the slide and coverglass and are not as thick as a spindle in typical cell culture experiments,

3. Does Rango-2 expression alter the steepness of Ran gradient? Are there controls to infer Rango-2 induced differences across the dataset?

We do not have any direct evidence to show that the Rango-2 biosensor itself causes a change in the steepness of the gradient. Recall that Rango-2 is only the importin beta binding domain of importin alpha, which forms a small 5 nm rod when bound to importin beta, fused to YPet and CyPet, and is the third generation of Petr Kalab's RanGTP gradient biosensors that he used in extracts (Science, 2002) and in cells (Nature, 2006). Rango-2 is not expected to change the production of RanGTP nor the RanGTP gradient based on these earlier studies, as perturbation of the Ran gradient or RanGTP production produced the expected FLIM or FRET results. In addition, there were no spindle defects observed with the biosensor alone. Furthermore, a similar biosensor was used to show that changes in the RanGTP gradient could be observed in cells with increased chromatin content (Hasegawa, 2013).

4. The proposed impact on cancer cells could be highlighted in the summary section - this is a clinically impactful idea that emerges from this basic biology work. It could potentially be easily tested by exogenously overexpressing XCTK2 mutant (for example the tail domain mutant) in a cell line exhibiting centrosome clustering defect. This line of experiment(s) is/are not critical for the current manuscript but will help bolster their proposal on centrosome clustering in cancer cells.

We agree that this would be a nice experiment to bolster our model of how cancer cells use an enhanced Ran gradient to cluster their centrosomes through enhanced Kinesin-14 activity. Unfortunately, there are some technical issues with this experiment because overexpression of the NLS mutant in cells is lethal due to excessive bundling of MTs in the cytoplasm (Cai, 2009). We hope to garner some evidence toward this idea by determining the relative contributions of parallel and anti-parallel MT cross-linking by determining whether there are two MT binding domains in the tail.

5. Figure 5B is somewhat unclear - Figures 5A and 5C make clear points and so Figure 5B could be omitted or presented in the context of the spindle.

We agree that Figure 5B is somewhat redundant to 5A and 5C (now Fig. 5D-E), but we chose not to include the gradient in Figure 5A to simplify the figure to highlight the interacting motors. Instead, the

gradient is included on the spindle in our big picture model in 5C (now Fig. 5 E). To reduce the redundancy, we have removed the delineation between 5A and 5B (now Fig. 5 D) and made changes to the figure legend in the hope that this appears less redundant.

October 30, 2019

RE: JCB Manuscript #201906045R

Dr. Claire E Walczak
Indiana School of Medicine - Bloomington
Medical Sciences
915 E. 3rd St. MY 262
Bloomington, IN 47405

Dear Claire:

Thank you for submitting your revised manuscript entitled "RanGTP induces an effector gradient of XCTK2 and importin α/β for spindle microtubule cross-linking". We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

First, if we may, there are a couple of items that we'd like for you to attend to in the final revision:

Using different nomenclatures to denote the same fusion protein in the main text vs. figures is confusing. I understand that YPet-XCTK2-ΔNLS is quite long and not easily pronounceable. However, the same identifier needs to be used throughout the manuscript.

A related issue is the clarity of cartoons presented in Fig.1 A-C. In my opinion, these panels would greatly benefit from a detailed graphic legend. There are numerous spheres and ovals shown in multiple colors but there is no key to the moieties represented by each element. Even for a person familiar with FRET as well as with the composition of kinesins, understanding that yellow spheres within 'YXCTK2' molecule represent YPet while red/magenta ovals are globular N-terminal domains of XCTK2 requires a serious effort. Similarly, a reader not familiar with Importin would have difficult time figuring out that Importin A is represented by blue spheres while Importin B is a green oval. A graphic legend added to the first panel would solve this problem.

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

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, http://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

1) Text limits: Character count for Reports is < 20,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, and acknowledgments. Count does not include materials and methods, figure legends, references, tables, or supplemental legends. You are currently a bit over this limit but we should be able to give you the extra space this time.

2) Figure formatting: Scale bars must be present on all microscopy images, including inset

magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.

3) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (both in the figure legend itself and in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, since you used parametric tests in your study (e.g. t-tests, ANOVA, etc.), you should have first determined whether the data was normally distributed before selecting that test. Currently, you note in the methods that "ANOVAs and two-tailed Welch's or Student's t-tests were performed in Prism based on the normality of the samples." However, please indicate precisely how you tested the data for normality. If you did not test for normality, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

4) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions (at least in brief) in the text for readers who may not have access to referenced manuscripts. The text should not refer to methods "...as previously described."

5) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies.

6) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
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b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. imaging medium
e. Fluorochromes
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g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

7) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

8) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Reports may have up to 3 supplemental figures. At the moment, you are below this limit but please bear it in mind when revising.
Please also note that tables, like figures, should be provided as individual, editable files.
A summary of all supplemental material should appear at the end of the Materials and methods section.

9) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

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Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

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Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

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