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Structure of the RZZ complex and molecular basis of Spindly-driven corona assembly at human kinetochores

Tobias Raisch, Giuseppe Ciossani, Ennio d'Amico, Verena Cmentowski, Sara Carmignani, Stefano Maffini, Felipe Merino, Sabine Wohlgemuth, Ingrid Vetter, Stefan Raunser, and Andrea Musacchio
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Corresponding author(s): Andrea Musacchio (andrea.musacchio@mpi-dortmund.mpg.de) , Ingrid Vetter (ingrid.vetter@mpi-dortmund.mpg.de), Stefan Raunser (stefan.raunser@mpi-dortmund.mpg.de)

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Dear Andrea,

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by two referees and their comments are provided below. As you can see both referees appreciate the analysis and find that the study makes an important contribution to the field.

I am still waiting to hear back from a third referee who had agreed to review the paper. I would like to give the referee this week to return the report before getting back to you with the final decision. However, given the positive comments of the two referees, I would like to ask you to start thinking about carrying out the needed revisions. Referee #3 brings up some valid points that would be good to experimentally address. Would be helpful to discuss these further either via email or a video call.

I will get back to you end of this week with the final decision

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

I have attached a PDF file with helpful tips on how to prepare the revised version.

Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (24th Apr 2022). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

https://emboj.msubmit.net/cgi-bin/main.plex

Referee #1:

In the manuscript entitled "Structure of the RZZ complex and molecular basis of Spindly-driven corona assembly at human kinetochores" Raisch and colleagues provide an improved, high-resolution cryo-EM structure of the RZZ complex that reveals a more detailed insight into the interactions between the RZZ subunits and into a farnesyl-binding site required for binding of farnesylated Spindly. Moreover, the authors developed an in-vitro reconstitution assay to study the mechanisms of filament nucleation of the RZZS complex that was shown to be essential for assembly and expansion of filamentous corona at the outer layer of kinetochores during early mitosis. This assay (showing high correlation with in cellulo corona assembly) confirmed earlier reports but also provided new insight on the importance of MPS1 kinase-mediated phosphorylation of Rod and farnesylation of Spindly in assembly of filamentous corona at the kinetochore. The authors propose a model in which MPS1-mediated phosphorylation release Rod from an autoinhibited state, allowing the filament assembly. Finally, they identified a short region in Spindly that encompasses 31 residues and is essential for its kinetochore localization and corona expansion. The data showed in this study are of the highest quality and the claims are convincing. The assembly and expansion of filamentous corona during early mitosis is essential for proper chromosome movements and accurate chromosome segregation, making this study of interest for a broader group of readers. The in vitro polymerization assay has a great potential to continue propelling the understanding of corona assembly/expansion and its importance in future.

Specific comments:

1) Lines 62-65: The sentence does not read well.
2) Lines 225, 279 and 406: The readers may benefit of having access to the data described as unpublished.
3) Figure 4 - Supplement 1H: Quantification of the signal would strengthen the claim that Aurora B inhibition fully abrogates the recruitment of RZZ and CENP-E.
4) The authors propose that Rod is autoinhibited and that MPS1-mediated phosphorylation releases this autoinhibition. It may be

of interest to discuss this in the context of previously proposed autoinhibition of Spindly (Sacristan et al, 2018), which was also proposed to be essential during corona assembly/expansion.
5) Line 384: It should read "co-existed" instead of "co-existing".
6) The in vitro polymerization assay revealed that N-terminal tagging of Spindly promotes the formation of curved filaments. Could the authors discuss how the N-terminal tagging affects this?
7) The study identified 31 residues of Spindly (276-306) as essential for its kinetochore localization and corona expansion. This goes in line with earlier work (Barisic et al, 2010) showing that deletion of 253 N-terminal residues results in localization of Spindly at expanded corona both in prometaphase and metaphase, whereas deletion of 293 N-terminal prevents Spindly localization at kinetochores. Discussing these two sets of data may suggest even shorter region as essential for kinetochore localization and corona expansion (276-293).

Referee #3:

The spindle assembly checkpoint (SAC) ensures the accuracy of chromosome segregation and genome stability. SAC signaling is initiated on unattached kinetochores, a multilayered macromolecular assembly rooted in centromeric chromatin. The ROD-Zwilch-ZW10 (RZZ) complex assembles into the fibrous corona that projects from the outer kinetochore. On the one hand, the fibrous corona promotes microtubule capture at least in part by recruiting microtubule motors, such as CENP-E. On the other hand, the fibrous corona enhances SAC signaling by providing the docking site for the MAD1-MAD2 core complex, which is in turn required for formation of the mitotic checkpoint complex (MCC). Despite its many functions in mitosis and extensive efforts from many labs studying it, the assembly mechanism of the fibrous corona remains poorly understood.

In this manuscript, Raisch et al. report the medium-resolution (3.9 Å) cryo-EM structure of the human RZZ complex, which revealed important structural features of the complex. Subsequent in vitro reconstitution and cellular experiments, guided by the structure and by AlphaFold2-based modeling, further identify additional requirements (e.g. Spindly farnesylation and ROD phosphorylation by MPS1) for filament formation by the RZZ-Spindly complex. Although it would have been more gratifying to learn the salient structural features of the RZZ-Spindly polymer and to understand how the dimeric RZZ transitions into the fibral form, this study nonetheless represents an important step forward. It provides key insight into the assembly of the fibrous corona and lays the foundation for further characterization of the incorporation of additional effector components into the corona, including CENP-E and MAD1-MAD2. Publication of this technically superb study is highly recommended.

The following points need to be addressed prior to publication.

Major points
(1) The interactions between ROD and Spindly were modeled by AlphaFold2. Do residues neighboring C602 of Spindly contribute to binding to ROD? If so, can the authors test this experimentally to further support their model, assuming that the mutations do not perturb farnesylation.

(2) The authors argue that phosphorylation of Thr13 and Ser15 by MPS1 would restructure the N-terminal region of ROD, relieving its auto-inhibition and allowing interactions required for the nucleation and growth of RZZS filaments. A major foundation of this hypothesis is again the AlphaFold2-based model of the ROD N-terminal domain. Is it possible to build a FRET-based sensor that directly probes the MPS1-induced (or the phospho-mimicking mutant) conformational change involving the N-terminal 29 residues of ROD?

(3) The authors speculated that the RZZS rings consisted of a staggered filament of "individual RZZS complexes". Do "individual RZZS complexes" refer to the 2:2:2 RZZ hexamers or 1:1:1 RZZ trimers? A staggered array of 2:2:2 RZZ hexamers would be predicted to have segments that are thicker than 11 nm. Please clarify.

Minor points

(1) In Figure 2F, the two ZW10 molecules are both labeled "ZW10-A". The light blue one should be "ZW10-B".

(2) Asn122 and Ser193 in the farnesyl group binding site of ROD were mentioned in the text but not shown in Figure 3E-F.

The complete list of differences between the revised manuscript and the original submission is appended at the end of this file. During revision of the manuscript, we realized that the heat-induced rings presented in the Figure 6D had been incorrectly labelled as ^{mCh}RZZ^{mCh}S^{F} while they were obtained with ^{mCh}RZZ^{GFP}S^{F}. We have corrected the figure accordingly. The conclusions of the experiment remain unaltered, as the heat-induced and MPS1-induced rings have essentially identical sizes regardless of the fluorophore used (see also comparison in panel 6B)

Referee #1:

In the manuscript entitled "Structure of the RZZ complex and molecular basis of Spindly-driven corona assembly at human kinetochores" Raisch and colleagues provide an improved, high-resolution cryo-EM structure of the RZZ complex that reveals a more detailed insight into the interactions between the RZZ subunits and into a farnesyl-binding site required for binding of farnesylated Spindly. Moreover, the authors developed an in-vitro reconstitution assay to study the mechanisms of filament nucleation of the RZZS complex that was shown to be essential for assembly and expansion of filamentous corona at the outer layer of kinetochores during early mitosis. This assay (showing high correlation with in cellulo corona assembly) confirmed earlier reports but also provided new insight on the importance of MPS1 kinase-mediated phosphorylation of Rod and farnesylation of Spindly in assembly of filamentous corona at the kinetochore. The authors propose a model in which MPS1-mediated phosphorylation release Rod from an autoinhibited state, allowing the filament assembly. Finally, they identified a short region in Spindly that encompasses 31 residues and is essential for its kinetochore localization and corona expansion. The data showed in this study are of the highest quality and the claims are convincing. The assembly and expansion of filamentous corona during early mitosis is essential for proper chromosome movements and accurate chromosome segregation, making this study of interest for a broader group of readers. The in vitro polymerization assay has a great potential to continue propelling the understanding of corona assembly/expansion and its importance in future.

We are very grateful to the reviewer for a positive assessment of our work and for several helpful suggestions.

Specific comments:

1) Lines 62-65: The sentence does not read well.

We agree and have rephrased the sentence.

2) Lines 225, 279 and 406: The readers may benefit of having access to the data described as unpublished.

We agree with the reviewer and have now included the data originally marked as "unpublished results" for the statements on line 279 and 406. Specifically, we have added a new panel EV3A for the data originally discussed on line 279. We have also added new EM micrographs of the rings in ice as panel Figure EV5F.

Our "(unpublished results)" statement on line 225 referred instead to the inability of AF2 and its variants (Colabfold and Multi) to identify a ZW10 dimer like the one observed in the RZZ complex. As documenting this would occupy a full supplementary figure, we have decided not to include these data in the manuscript, and we rather present them at the end of this rebuttal letter. On the other hand, we feel that the exceptionally accurate AF2 prediction of ZW10 by AF2 is worth mentioning and we have added panel Appendix Figure S1E to document this.

3) Figure 4 - Supplement 1H: Quantification of the signal would strengthen the claim that Aurora B inhibition fully abrogates the recruitment of RZZ and CENP-E.

We agree with the reviewer and have now included a quantification of the signals in the new panel EV3J.

4) The authors propose that Rod is autoinhibited and that MPS1-mediated phosphorylation releases this autoinhibition. It may be of interest to discuss this in the context of previously proposed autoinhibition of Spindly (Sacristan et al, 2018), which was also proposed to be essential during corona assembly/expansion.

We agree with the reviewer and have now modified the Discussion to incorporate a new paragraph focusing on this interesting question. A few sentences were reshuffled within the Discussion to accommodate this.

5) Line 384: It should read "co-existed" instead of "co-existing".

Corrected

6) The in vitro polymerization assay revealed that N-terminal tagging of Spindly promotes the formation of curved filaments. Could the authors discuss how the N-terminal tagging affects this?

In the absence of a molecular model for RZZS polymerization, we prefer to refrain from speculating on the molecular basis of this phenomenon. However, we write that tagging prevents filament formation "likely because it inhibits the "lateral" interaction of RZZS protomers that enables the formation of sheets."

7) The study identified 31 residues of Spindly (276-306) as essential for its kinetochore localization and corona expansion. This goes in line with earlier work (Barisic et al, 2010) showing that deletion of 253 N-terminal residues results in localization of Spindly at expanded corona both in prometaphase and metaphase, whereas deletion of 293 N-terminal prevents Spindly localization at kinetochores. Discussing these two sets of data may suggest even shorter region as essential for kinetochore localization and corona expansion (276-293).

We apologize for overlooking the important information in the Barisic et al. 2010 paper, to which we now duly refer.

Referee #2:

In the absence of microtubule attachment, vertebrate kinetochores undergo a phosphodependent expansion of their outermost layer, forming the so-called fibrous corona. Corona formation enhances the speed and efficiency of microtubule attachment, and may also play a role in enhancing spindle assembly checkpoint (SAC) signaling and inhibition of anaphase onset. Recent work has established the Rod-Zw10-Zwilch (RZZ) complex, Spindly, and Mps1 kinase as the key building blocks and activators of corona assembly, with a specific role of N-terminal phosphorylation of Rod. However this phosphodependent process has not been reconstituted in vitro.

Here Rasich et al generate a high-resolution structural model of the RZZ-Spindly (RZZ-S) complex via cryo-EM, 3D modeling, and AI-assisted structure prediction. Through biochemical reconstitution, the authors establish the requirements for (and roles of) specific domains in RZZ-

S for Mps1-dependent oligomerization. Because of the congruence of biochemical and genetic/cell biological dependencies, there is a strong (if circumstantial) argument for equating oligomerization in vitro with corona formation in vivo. The authors go on to propose that RZZ-S protomers are released from an auto-inhibited state upon Mps1 phosphorylation.

The study addresses a topic of considerable importance and interest in the mitosis field. The experiments are well executed and generally well interpreted. Taken together with previous work in this area, the study provides a more detailed and useful picture of RZZ-S protomer architecture and oligomerization under the control of Mps1. I have some suggestions for improving accuracy and clarity of this worthwhile study.

We are very grateful to the reviewer for a positive assessment of our work and for several helpful suggestions

1. At several points the authors make the claim that Mps1 inhibition blocks RZZ-dependent kinetochore expansion but not RZZ's own kinetochore recruitment. I agree that these are separable via the N-terminal phosphorylation site mutations in Rod, but Santaguida et al. (2010) and Maciejowski et al. (2010) both report that Mps1 inhibition evicts RZZ subunits from kinetochores, to a degree similar to eviction of Mad1. Since this claim is parenthetical to the main points of the study, I suggest eliminating it.

We thank the reviewer for pointing out this difference in the penetrance of MPS1 inhibition, and we agree with the reviewer that Santaguida *et al.* 2010 reported a more severe effect on RZZ and Spindly recruitment from inhibiting MPS1 than observed in our new experiments. We also agree with the reviewer that the effects are separable. While we are unclear about the sources of the difference, we feel it is important to communicate this clearly and we now write: "These effects of Reversine on RZZ and Spindly recruitment are entirely consistent with a recent report (Rodriguez-Rodriguez et al., 2018) but milder than we had reported previously (Santaguida et al., 2010), arguably a consequence of improved experimental conditions and quantification procedures."

2. "The inability of the T13A/S15A mutant of mChRZZSF to form fibers did not reflect a requirement of these residues in fiber assembly, because fibers of this mutant were observed after mildly heating the sample to 30" (lines 342-344). I found this sentence confusing and potentially interpretable as contradicting the other results/claims in the paper. The data show that the phosphorylation state of Rod is important for fiber assembly. Heat treatment bypassed this dependence, but this result does not mean there is no dependence. This needs to be reworded to eliminate confusion.

We agree with the reviewer that the sentence was potentially misleading. After introducing new experiments with a Δ26 mutant, described in our response to Reviewer 3, we have now extensively re-written this section, indicating as clearly as possible that we believe T13 and T15 to be very important for filament nucleation.

3. The kinetochore microscopy panels and insets in Figures 4 and 7 are very dim and have too little contrast. The insets are also too small. Please correct/modify.

We have now corrected both the panels and the insets.

Referee #3:

The spindle assembly checkpoint (SAC) ensures the accuracy of chromosome segregation and genome stability. SAC signaling is initiated on unattached kinetochores, a multilayered macromolecular assembly rooted in centromeric chromatin. The ROD-Zwilch-ZW10 (RZZ) complex assembles into the fibrous corona that projects from the outer kinetochore. On the one hand, the fibrous corona promotes microtubule capture at least in part by recruiting microtubule motors, such as CENP-E. On the other hand, the fibrous corona enhances SAC signaling by providing the docking site for the MAD1-MAD2 core complex, which is in turn required for formation of the mitotic checkpoint complex (MCC). Despite its many functions in mitosis and extensive efforts from many labs studying it, the assembly mechanism of the fibrous corona remains poorly understood.

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We are very grateful to the reviewer for a positive assessment of our work and for several helpful suggestions

The following points need to be addressed prior to publication.

Major points

(1) The interactions between ROD and Spindly were modeled by AlphaFold2. Do residues neighboring C602 of Spindly contribute to binding to ROD? If so, can the authors test this experimentally to further support their model, assuming that the mutations do not perturb farnesylation.

We now present results with four new point mutants of Spindly affecting residues predicted to interact with ROD a few residues away from the farnesyl-binding pocket (new panels A-B in Figure EV2). The new mutants are fully in line with the modelling, and decrease, to varying degrees, the interaction of Spindly with RZZ.

(2) The authors argue that phosphorylation of Thr13 and Ser15 by MPS1 would restructure the N-terminal region of ROD, relieving its auto-inhibition and allowing interactions required for the nucleation and growth of RZZS filaments. A major foundation of this hypothesis is again the AlphaFold2-based model of the ROD N-terminal domain. Is it possible to build a FRET-based sensor that directly probes the MPS1-induced (or the phospho-mimicking mutant) conformational change involving the N-terminal 29 residues of ROD?

We have carefully considered the reviewer's suggestion to build a FRET sensor, but feel that this may require a very significant effort, probably beyond what might be expected for a paper that is already very data-rich. To build such a sensor in ROD, we would have to co-express our subunits in insect cells, probably labelling the N-terminus of ROD with a fluorophore and an unknown internal site in the ROD beta-propeller (e.g. in a loop) with another one. This is a non-trivial effort because we would have to try various sites, internal to the beta-propeller, compatible with insertion of a fluorophore. Furthermore, even if we were successful, MPS1 phosphorylation will cause the

N-terminal region to become loose, possibly reducing the FRET signal, but because the loosening of the N-terminus will coincide with assembly of the filament, we will have to exclude that the removed N-terminus, with its fluorophore, lands near the second fluorophore on a different molecule. Furthermore, the new internal fluorophore should not interfere with oligomerisation. In other words, we feel that this is a very complex experiment and that it has a fairly high chance of failing. Had it been bacterial recombinant expression, we would have been more positive about trying, but with insect cell expression, we are afraid that this may require several months of preparatory work without a guarantee of success.

We have nevertheless further investigated this question. In the original manuscript, we had described experiments with $^{mCh}ROD^{\Delta 15}$: "An $^{mCh}RZZS^{F}$ complex containing a ^{mCh}ROD deletion mutant lacking the N-terminal 15 residues ($^{mCh}ROD^{\Delta 15}$) was insensitive to MPS1 phosphorylation, but, like the T13A/S15A mutant, formed fibers when heat-treated (Figure 5F). Thus, $^{mCh}ROD^{\Delta 15}$ remains auto-inhibited, probably due to the residual capping of blade 6 with the β6e strand, which we speculate to be crucially required for fiber assembly."

To test this prediction, at the time of the original submission we had already begun to prepare a longer deletion mutant in view of a possible revision. We have now studied this mutant, $^{mCh}ROD^{\Delta 26}$, which we present in an enlarged panel (Figure 5F). Contrary to our prediction, this mutant behaved indistinguishably from $^{mCh}ROD^{\Delta 15}$. This result implies that the N-terminal region is not strictly required for filament assembly, but that is also not a simple gatekeeper whose phosphorylation unleashes rapid filament assembly, as the rapid assembly triggered by phosphorylation is not recapitulated by its deletion. We have therefore rephrased this section accordingly. We feel that further characterization of the oligomerization mechanism will require additional work that goes beyond the scope of the present manuscript.

(3) The authors speculated that the RZZS rings consisted of a staggered filament of "individual RZZS complexes". Do "individual RZZS complexes" refer to the 2:2:2 RZZ hexamers or 1:1:1 RZZ trimers? A staggered array of 2:2:2 RZZ hexamers would be predicted to have segments that are thicker than 11 nm. Please clarify.

Staggering of hexamers along the filament may be expected to result in filaments with a cross-section of approximately 11 nm x 22 nm (1 unit x 2 units). So, staggered filaments would be expected to remain 11 nm if viewed perpendicularly to the thin side of the filament.

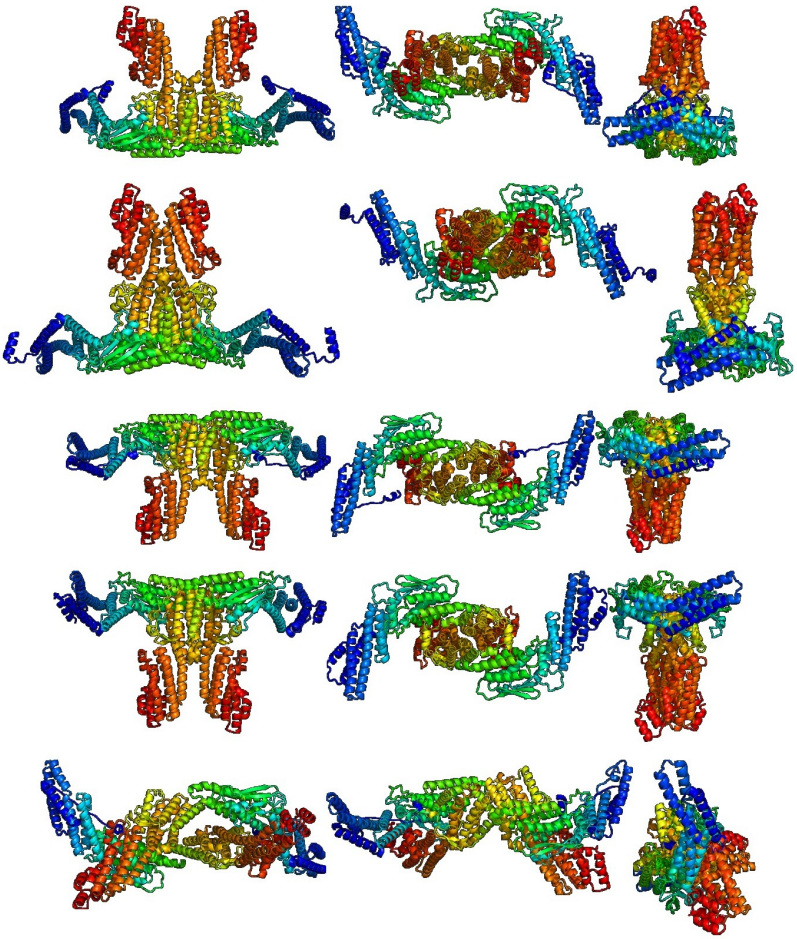
Minor points

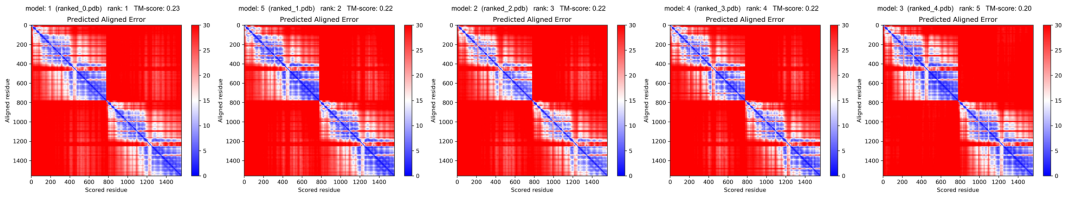
(1) In Figure 2F, the two ZW10 molecules are both labeled "ZW10-A". The light blue one should be "ZW10-B".

Corrected

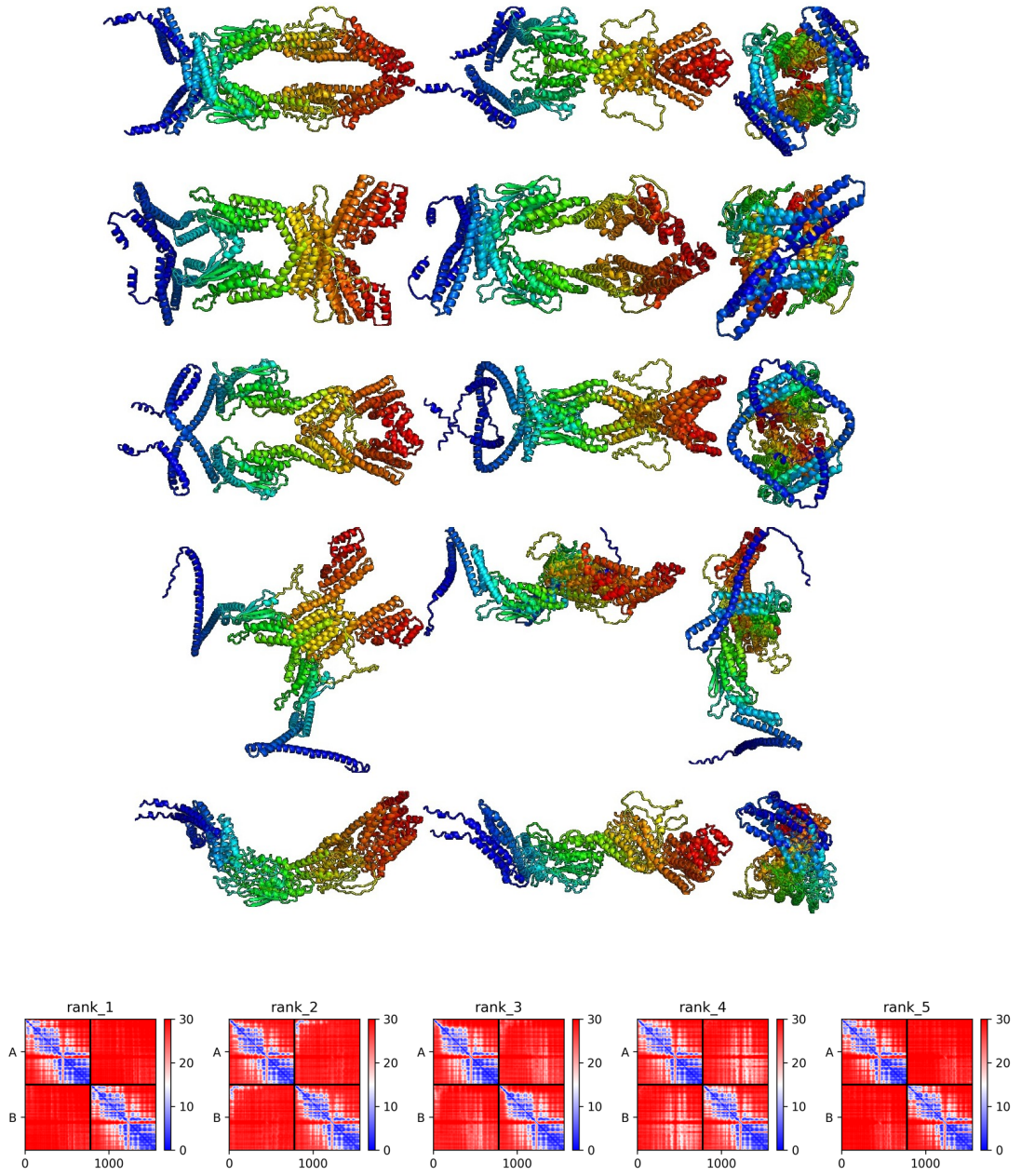
(2) Asn122 and Ser193 in the farnesyl group binding site of ROD were mentioned in the text but not shown in Figure 3E-F.

We thank the reviewer for pointing this out. We have now added labels for the two residues, whose side chains were already shown but not labelled.





Five models of ZW10 dimers from AF2 Multimer (*top*; each model is displayed from three orthogonal views and colored from N- to C-terminus in rainbow colors from blue to red) and corresponding PAE plots (*bottom*). Sequence numbering in the PAE plots correspond to the two tandem ZW10 sequences



Five models of ZW10 dimers (A and B) from AF2 ColabFold (*top*; each model is displayed from three orthogonal views and colored from N- to C-terminus in rainbow colors from blue to red) and corresponding PAE plots (*bottom*).

Rodriguez-Rodriguez, J.A., C. Lewis, K.L. McKinley, V. Sikirzhytski, J. Corona, J. Maciejowski, A. Khodjakov, I.M. Cheeseman, and P.V. Jallepalli. 2018. Distinct Roles of RZZ and Bub1-KNL1 in Mitotic Checkpoint Signaling and Kinetochore Expansion. *Current biology : CB*. 28:3422-3429 e3425.

Santaguida, S., A. Tighe, A.M. D'Alise, S.S. Taylor, and A. Musacchio. 2010. Dissecting the role of MPS1 in chromosome biorientation and the spindle checkpoint through the small molecule inhibitor reversine. *The Journal of cell biology*. 190:73-87.

Dear Andrea,

Thank you for submitting your revised manuscript to The EMBO Journal.

Your revision has now been seen by referee #3 and as you can see below, this referee appreciates the introduced changes.

I am therefore pleased to let you know that we will accept the manuscript for publication here. Before sending you the formal acceptance letter there are just a few editorial points to sort out

- we can only have 3-5 keywords

- Please make sure that you make the PDB codes publicly available.

- We need a Disclosure Statement & Competing interest statement - this replaces our COI section. Please see guide to authors https://www.embopress.org/page/journal/14602075/authorguide#conflictsofinterestC

- The author contribution section should be moved, along with the Acknowledgements, to after the Materials and Methods section. Only initials should be used.

- Figure callouts are missing for Fig 4F and Fig EV1A-D.

- Our publisher has also done their pre-publication check on your manuscript. When you log into the manuscript submission system you will see the file "Data Edited Manuscript file". Please look at the word file and the comments regarding the figure legends and respond to the issues.

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- I also need a summary figure for the synopsis. The size should be 550 wide by [200-400] high (pixels).

- Table EV1 needs the name correcting in the file itself.

- Fig 4H panel is missing from the figure.

That should be all. You can use the link below to upload the revised version.

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (5th Jun 2022). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

https://emboj.msubmit.net/cgi-bin/main.plex

Referee #3:

The authors have adequately addressed my concerns. Although they could not build FRET sensors to investigate the proposed

conformational changes, they provided good rationale for not being able to do so. I recommend the publication of this excellent study.

2nd Revision - Editorial Decision **11th Mar 2022**

Dear Andrea,

Thank you for submitting your revised manuscript the EMBO Journal. I have now had a chance to take a look at everything and all looks good!

I am therefore very pleased to accept the manuscript for publication here. Congratulations on a nice study!

Will you please make sure that you make the PBD codes accessibile?

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your manuscript.

Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for 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.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots 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. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship 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.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Yes	Materials and Methods

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Materials and Methods

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Materials and Methods

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Yes	Materials and Methods
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Not Applicable	
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes	We regularly test our laboratory cell lines for Mycoplasma infection

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Not Applicable	
Animal observed in or captured from the field: Provide species, sex, and age where possible.	Not Applicable	
Please detail **housing and husbandry conditions**.	Not Applicable	

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Acknowledgements

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Not Applicable	
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Not Applicable	
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Materials and Methods

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Figure legends
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Figure legends

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: 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.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Not Applicable	
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
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.	Not Applicable	
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.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	Data availability
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Yes	Main text, reference list (PDB files from other studies)