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Stable kinetochore-microtubule attachment requires loop-dependent Ndc80-Ndc80 binding

Soumitra Polley, Helen Müschenborn, Melina Terbeck, Anna De Antoni, Ingrid Vetter, Marileen Dogterom, Andrea Musacchio, Vladimir Volkov, and Pim Huis in 't Veld
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Dr. Pim J. Huis in 't Veld
Max Planck Institute of Molecular Physiology
Mechanistic Cell Biology
Otto Hahn Strasse 11
Dortmund 44227
Germany

5th Oct 2022

Re: EMBOJ-2022-112504
Stable kinetochore-microtubule attachment requires loop-dependent Ndc80-Ndc80 binding

Dear Pim, Vladimir, and Andrea,

Thank you again for submitting your study on Ndc80 loop-mediated functions to The EMBO Journal. I have now received reports from three expert referees, copied below for your information. As you will see, all referees acknowledge the potential importance of this work, and appreciate in particular the presented biochemical and biophysical experiments. At the same time, especially referees 1 and 2 remain unconvinced that the in vitro results are currently sufficiently well-connected with the cellular data, which the reviewers do not yet consider fully conclusive at this point. Consequently, all referees ask for more definitive evidence that loop-mediated Ndc80 clustering is important in cells, and for better insight into the seemingly separate loop function in checkpoint signaling.

Should you be able to adequately address these key concerns, and to extend the analyses in the direction suggested by the reviewers, we would be happy to pursue a revised version of the study further. Since the main issues recur in similar form throughout the three reports, addressing them experimentally and not just textually would in my view significantly strengthen the study, and it might therefore be helpful to discuss already during the early stages of your revision how this might be achieved. I would therefore invite you to carefully consider the reports together with your co-workers, and to send me a tentative point-by-point response via email, which could serve as the basis for further discussion via email or online call. I should add that we could also offer extension of the default three-months revision period if needed, with our 'scooping protection' (meaning that competing work appearing elsewhere in the meantime will not affect our considerations of your study) remaining of course valid also throughout this extension.

Detailed information on preparing, formatting and uploading a revised manuscript can be found below and in our Guide to Authors. Thank you again for the opportunity to consider this work for The EMBO Journal, and I look forward to hearing from you in due time.

With kind regards,

Hartmut

Hartmut Vodermaier, PhD
Senior Editor, The EMBO Journal
h.vodermaier@embojournal.org

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- size of the scale bars that are mandatory for all micrograph panels
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- the type error bars (e.g., S.E.M., S.D.)
- the number (n) and nature (biological or technical replicate) of independent experiments underlying each data point

- Figures may not include error bars for experiments with n<3; scatter plots showing individual data points should be used instead.

3) Revised manuscript text (including main tables, and figure legends for main and EV figures) has to be submitted as editable text file (e.g., .docx format). We encourage highlighting of changes (e.g., via text color) for the referees' reference.

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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 (3rd Jan 2023). 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:

Link Not Available

Referee #1:

the authors examine potential cooperation between Ndc80 complexes, as a means to understand how ~250 copies bound to ~10 microtubules at a kinetochore behave. Using AF-multimer, they generate a high confidence structure, and use it to focus in the loop region for further functional characterisation. They show that Loopless Ndc80 binds microtubules in vitro but does not seem to cluster with other molecules like WT does. Furthermore, trimerised Loopless Ndc80 displayed higher diffusion rates on microtubules in vitro compared to WT, and single molecule biophysical experiments show that loopless Ndc80 cannot support load-bearing attachment to microtubules as well as WT can. In cells, Loopless Ndc80 or a minimal mutant mimicking loopless Ndc80 (M5) rescues Ndc80 depletion in the SAC response but not in generating stable interactions with microtubules. The identification of M5 lead the authors to show that M5 trimer displayed much reduced affinity for WT monomer on microtubules, suggesting the loop is required for multimerising Ndc80 molecules. To more directly test if loop-mediated interactions between ndc80 complexes is needed for function, the authors use antibodies that bind in or close to the loop region. One of them indeed causes substantial reductions in diffusion on microtubules. When injected into cells, however, the antibody could not restore congression in loopless cells, but did affect the ability of cell expressing loopless ndc80 to mount a robust SAC response.

This is an insightful study on molecular properties of Ndc80 required for kinetochore-microtubule attachments. The biochemical and biophysical experiments are solid and of high quality and show the importance of the loop for multimerisation and thereby for formation of load-bearing attachments between kinetochores and microtubules. I found the antibody and cell experiments a

little less convincing however:

A lot of the conclusions are based on the Ab849, but it is unclear to me what it is doing. On the one hand, it is argued that it can x-link Ndc80 molecules and on the other that it prevents some sort of loop-proximal SAC function. Here is where it becomes quite confusing. Initially, the authors present the Ab849 as a tool to restore cross-linking interactions to loopless mutants. If this x-linking were essential for function, the Ab should restore congression in cells, but is doesn't. How come? The authors then use the antibody in SAC experiments, and show that it affects the SAC in noco-treated cells but only when those cells express loop mutants. In other words, the Ab does more than cause x-linking, because otherwise there should be no difference between WT (naturally x-linked) and mutants (artificially x-linked). Here is where the initially straightforward story on the loop takes a turn into a confusing direction that deviates from the loop and focusses on a loop-proximal region bound by the Ab, but without satisfying answers. It is therefore crucial to show what Ab849 is doing. First, where is the evidence that it causes cross-linking of Ndc80 molecules to begin with? Simply concluding this because it reduces diffusion of Ndc80 complexes on microtubules is circular reasoning and therefore insufficient. Second, is Ndc80 its only antigen in cells? Perhaps not, which would confound interpretations. Third, if the Ab inhibits some loop-proximal region important for the SAC, one would predict that mutating that region in the context of loop mutants should impact the SAC, alleviating the need to rely solely on the Ab experiment. Finally, a minor comment: i don't quite get how data in 7A is presented: in line 319-321 much is made of showing that loop-mediated clustering does more than simply impact the AuroraB-error correction pathway, but isn't that already shown by all the in vitro experiments (in which tails are not phosphorylated)? Similarly, the data is said to show that 9A does more than satisfy the SAC (lines 334-336) but in my understanding of the cited papers, that is exactly what they proposed as well: that 9A binding to MTs overrides the SAC.

Referee #2:

In this work, Polley et al. investigate the role of the loop region of Ndc80 (a structured region at a break in an extended coiled-coil region) in the NDC80 complex's function. They demonstrate that the loop mediates microtubule-dependent homotypic interactions between NDC80 complexes in vitro, and that these interactions impact NDC80:microtubule binding stability. In addition, their experiments suggest that the Ndc80 loop has a role in proper kinetochore function and chromosome segregation in cells, consistent with prior work. Although the in vitro experiments suggest a novel and interesting role of the Ndc80 loop in NDC80 complex oligomerization and microtubule interactions, their in vitro and in vivo work seem largely disconnected and they fail to convincingly tie the oligomerization to NDC80 function in cells. In particular, there are multiple alternate explanations that could explain the cellular phenotypes that they have not excluded. At a minimum, the authors need to use extensive caution in interpreting these results, and should substantially rephrase their conclusions throughout the paper, although additional data could allow them to make stronger statements. In addition, although they mention prior work on the Ndc80 loop in their discussion section, they do not acknowledge these alternate possibilities throughout the text in a way that leads to a broad feeling of them overstating their conclusions.

Major Points
1. The authors fail to link the Ndc80 oligomerization behavior to the cellular phenotype. It is clear from their work that the loop enables oligomerization in vitro and that the loop is necessary for proper chromosome congression in vivo, but there is no direct evidence that the congression defect is caused by a failure to oligomerize. They also do not test some reasonable alternative hypotheses, including evaluating Ndc80 phosphorylation status (for example, using phospho-antibodies) or analyzing the localization of other outer kinetochore proteins, such as the SKA complex.
2. The mutant that they chose to explore to analyze Ndc80 function in cells (M5) phenocopies ΔLoop in cells, but M5 trimers diffuse more similarly to wild-type than to ΔLoop. This suggests that its microtubule binding activity isn't that different from wild-type such that unstable microtubule binding caused by an oligomerization defect is not a common cause of ΔLoop and M5's phenotypes. This casts the authors' hypothesis into doubt.
3. One way to explore this question further is to pursue additional mutants. They generated 11 mutants and observed several distinct phenotypes, but only characterized one. Their results suggest that the loop has at least two roles: one in oligomerization, and one in the SAC (although this may be related to microtubule binding indirectly). They could characterize those mutants in vitro to determine which ones have an oligomerization defects. If any of them have an oligomerization defect but don't cause a phenotype, it would suggest that the oligomerization defects does not cause the phenotype. If all mutants that have an oligomerization defect also fail to congress their chromosomes, it would provide stronger support that oligomerization is important.
4. An alternative approach would be to force oligomerization in cells to rescue ΔLoop mutants. They attempted to do this with an antibody, but this approach has several caveats. First, IgG should only cause dimerization, which is likely not the case for the Ndc80 loop based on their in vitro results. While dimerization may be sufficient to rescue the microtubule-binding phenotype of a trimer in vitro, it might not be sufficient for a kinetochore with hundreds of NDC80 complexes. Second, antibodies frequently impair the functions of their targets in living systems (for example, Deluca et al. 2006 for the Ndc80 complex), as is likely the case here making their in vivo antibody experiments difficult to interpret. Finally, they don't show that these antibodies drive oligomerization in cells. Even if the antibody did not impair wild-type function, the authors would need to show that it actually oligomerizes NDC80 complexes in cells for these results to be interpretable. Because of these caveats, it would be preferable to attempt a similar experiment using an alternative approach. One possibility is oligomerizing tags (similar to Hara et al. 2022

BioRxiv), possibly on a different subunit of the complex that is less sensitive to tagging. However, I recognize that these experiments represent a substantial investment - another reason why using much more caution in their conclusions based on their existing data is important.

Minor Points
1. It could be worth showing that the fold of the NDC80 loop is dependent on a disulfide bond by adding a reducing agent.
2. Line 219: "in presence" should be "in the presence."
3. In Figure 3A, "bead restores with growing MT" is unclear. "Bead returns to center of laser" would be more clear.
4. In figure 4E, the colors are very hard to distinguish for colorblind people. Red and Blue or Green and blue would be better.
5. In figure 4E, it would be nice to put the amino acid numbers at the beginning and end of the sequences labeled.
6. In line 266, Figure 5B is referenced alone. 5B-D should be referenced.
7. In line 275, Figures 5D and Figure S6 are referenced. It should also reference figure 5B.
8. In Figure 5B, it needs to be made clear that the x axis is the position along the microtubule. It would also be nice to have axis labels on the first graph to make it clear that it's a kymograph.
9. In Figure 6A, indicate which part of the sequence corresponds to which antibody.
10. It isn't clear how the section "Loop-mediated mitotic arrest involves multiple phospho-signaling pathways" relates to the rest of the story.
11. In Figure S8, add DAPI greyscale to show congression defects more clearly.
12. In line 372, the authors state that "the region immediately preceding the loop [...] [is] important for SAC signaling." This is an overinterpretation. AB-849 could cause the phenotype by steric hindrance or by stabilizing an dysfunctional conformation of the complex even if the region it binds is not directly involved in SAC function.
13. In line 386-388, the authors state that "the NDC80 loop promotes Ndc80-Ndc80 interactions that are crucial to generate force resistant attachments." This is also an overinterpretation. They show that the loop is essential for force-resistant attachment in vitro. They do not demonstrate that it is the oligomerization function of the loop that is essential for force-resistant attachments.
14. In lines 388-390, the authors state that "Our observations also suggest that interactions between adjacent Ndc80 complexes may signal the establishment of load-bearing kinetochore-microtubule attachments and silence the SAC." Nothing that they have shown supports this model.
15. The word "nematic" (line 431) is obscure and distracting to the reader - use simpler language.

Referee #3:

This is an interesting study aiming to explore a new role of the Ndc80 loop in kinetochore-microtubule interactions. The authors raised a hypothesis that the loop-mediated Ndc80 clustering is critical for proper kinetochore-microtubule interactions. To test this hypothesis, the authors performed a series of biochemical, biophysical and cell-biological experiments. Overall, this is a good study and the results largely support the authors' conclusions. The findings could potentially contribute to the mechanistical understanding of how proper kinetochore-microtubule interactions are achieved. Several points need to be addressed before moving forward to the next step.

1. The major point in this study is loop mediated Ndc80 clustering. The direct evidence for it is the results from Figure J and K. This clustering seems to be present only in the presence of microtubules. In the tube without microtubules, Ndc80 full-length and delta-L behave almost same in size-exclusion chromatography (suppl. Figure 3A). These observations suggest that binding of Ndc80 WT to microtubules might do "something" to allow Ndc80 clustering, whereas absence of loop fails to do so. I think that this possibility needs to be taken into account in the discussion.

2. Although the idea of loop mediated Ndc80 clustering is interesting, how the loop does so is unclear. Several experiments could be performed to potentially provide a clue. Firstly, might the loop alone be oligomerized in vitro? Secondly, how are Ska and SKAP/Astrin kinetochore recruitment affected in M1, M2, M5, M6, and M10 mutants.

Minor points:

3. In Figure 5A, it seems that Ndc80-M5 is less recruited to kinetochores. Quantification is needed.

4. In Figure 8, the legend title should be "Synergistic contributions of the Ndc80.....". In Supple. Figure 7, the "B" is misplaced.

5th Oct 2022

Re: EMBOJ-2022-112504
Stable kinetochore-microtubule attachment requires loop-dependent Ndc80-Ndc80 binding

Dear Pim, Vladimir, and Andrea,

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With kind regards,
Hartmut

April 5th, 2023
Dortmund, Germany

Revision EMBOJ-2022-112504
Stable kinetochore-microtubule attachment requires loop-dependent Ndc80-Ndc80 binding

Dear Hartmut,

we thank you and the three reviewers for insightful and constructive comments.

We are pleased to hereby submit a revised version of our work with several new experiments to address the reviewers' concerns, and more specifically towards strengthening the connection between our *in vitro* and *in vivo* results and characterizing the function of the AB-849 antibody.

Most notably, we have considerably extended the biochemical and biological characterization of loop mutants. We have now identified two basic residues within the Ndc80 loop region that are essential for chromosome congression in cells and also for the clustering of Ndc80 on microtubules in a fully reconstituted setup (Figure 8A-B).

Experiments with a new set of mutants (mutated in the AB-849 epitope region) did not provide evidence for a direct involvement of the loop in the spindle assembly checkpoint signalling. This prompted us to revise our previous interpretations. To improve the clarity of our manuscript, we included these new results in EV Figure 5, together with the results formerly presented as Figure 7B, and shortened our description of these experiments in the main text.

We would also like to refer to a recent structural characterization of the loop region of Ndc80 by Jenni, Zahm, and Harrison (bioRxiv, November 2022, https://doi.org/10.1101/2022.11.09.515846; Open Biology, March 2023, https://doi.org/10.1098/rsob.220378), providing experimental support for our structural predictions.

Please find a detailed point-by-point response to the reviewer's comments below (in blue).

With our very best regards,

Andrea, Vladimir, and Pim

Referee #1:

the authors examine potential cooperation between Ndc80 complexes, as a means to understand how ~250 copies bound to ~10 microtubules at a kinetochore behave. Using AF-multimer, they generate a high confidence structure, and use it to focus in the loop region for further functional characterisation. They show that Loopless Ndc80 binds microtubules in vitro but does not seem to cluster with other molecules like WT does. Furthermore, trimerised Loopless Ndc80 displayed higher diffusion rates on microtubules in vitro compared to WT, and single molecule biophysical experiments show that loopless Ndc80 cannot support load-bearing attachment to microtubules as well as WT can. In cells, Loopless Ndc80 or a minimal mutant mimicking loopless Ndc80 (M5) rescues Ndc80 depletion in the SAC response but not in generating stable interactions with microtubules. The identification of M5 lead the authors to show that M5 trimer displayed much reduced affinity for WT monomer on microtubules, suggesting the loop is required for multimerising Ndc80 molecules. To more directly test if loop-mediated interactions between ndc80 complexes is needed for function, the authors use antibodies that bind in or close to the loop region. One of them indeed causes substantial reductions in diffusion on microtubules. When injected into cells, however, the antibody could not restore congression in loopless cells, but did affect the ability of cell expressing loopless ndc80 to mount a robust SAC response.

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We thank referee #1 for their positive evaluation of the main body of our work.

A lot of the conclusions are based on the Ab849, but it is unclear to me what it is doing. On the one hand, it is argued that it can x-link Ndc80 molecules and on the other that it prevents some sort of loop-proximal SAC function. Here is where it becomes quite confusing. Initially, the authors present the Ab849 as a tool to restore cross-linking interactions to loopless mutants. If this x-linking were essential for function, the Ab should restore congression in cells, but is doesn't. How come?

The authors then use the antibody in SAC experiments, and show that it affects the SAC in noco-treated cells but only when those cells express loop mutants. In other words, the Ab does more than cause x-linking, because otherwise there should be no difference between WT (naturally x-linked) and mutants (artificially x-linked). Here is where the initially straightforward story on the loop takes a turn into a confusing direction that deviates from the loop and focusses on a loop-proximal region bound by the Ab, but without satisfying answers. It is therefore crucial to show what Ab849 is doing.

We thank the reviewer for raising this important point. Following the reviewer's suggestion, we tested the effects of the AB-849 more thoroughly and tried to improve the interpretation of our results and the clarity of our manuscript.

Please find a point-by-point rebuttal below.

First, where is the evidence that it causes cross-linking of Ndc80 molecules to begin with? Simply concluding this because it reduces diffusion of Ndc80 complexes on microtubules is circular reasoning and therefore insufficient.

When loopless Ndc80 trimers and microtubules are mixed in the presence of AB-849, we see that Ndc80 trimers accumulate over time. This depends on the presence of the antibody, reflecting Ndc80-Ndc80 crosslinking between trimers. We added this analysis to our revised manuscript as **Appendix Figure 4**.

We find AB-induced crosslinking of Ndc80 arms a very likely explanation of the correlation between AB-Ndc80 colocalization and diffusion on the microtubule lattice (**Figure 6**). Neither the reduction of diffusion nor the accumulation of trimers over time was observed when loopless Ndc80 was exposed to AB-850, raised against the epitope missing in the loopless construct (**Figure 6** and **Appendix Figure**

4B). Providing further proof of Ndc80-Ndc80 crosslinking within a handful of Ndc80 complexes, with or without antibody, is a truly demanding task that we consider to go beyond the scope of this study, where we provide an already vast body of evidence in support of Ndc80 oligomerization and its importance.

Second, is Ndc80 its only antigen in cells? Perhaps not, which would confound interpretations.

To address the reviewer's concern, we now demonstrate by immunoblotting that Ndc80 is also the main antigen of AB-849 and AB-850 in a whole cell extract in HeLa cells (**EV Figure 5**).

Third, if the Ab inhibits some loop-proximal region important for the SAC, one would predict that mutating that region in the context of loop mutants should impact the SAC, alleviating the need to rely solely on the Ab experiment.

We thank the reviewer for raising this point. To address this question, we have generated Ndc80 constructs that are mutated in the AB-849 epitope region (in isolation or combined with loop mutants M5 and ΔLoop). We purified this set of six new mutants (M15-M20), electroporated them into cells after depletion of endogenous Ndc80, and tested their progress through mitosis and their SAC response in the absence and presence of nocodazole. These experiments did not provide evidence for a direct contribution of the AB-849 epitope region to SAC signalling (Suppl. Figure 8). In other words, while the antibody shows a synthetic negative effect on checkpoint signalling when combined with the loop mutants, mutating the epitope of the antibody does not result in the same effect. We suspect that the effect of the antibody may be due to a steric effect, rather than a direct effect on its binding epitope.

To improve the clarity of our manuscript, we propose to move the in vivo results with AB-849 and AB-850, formerly presented as **Figure 7B**, to **EV Figure 5**. Furthermore, we have drastically shortened our description of these experiments in the revised main text.

Finally, a minor comment: i don't quite get how data in 7A is presented: in line 319-321 much is made of showing that loop-mediated clustering does more than simply impact the AuroraB-error correction pathway, but isn't that already shown by all the in vitro experiments (in which tails are not phosphorylated)?

Similarly, the data is said to show that 9A does more than satisfy the SAC (lines 334-336) but in my understanding of the cited papers, that is exactly what they proposed as well: that 9A binding to MTs overrides the SAC.

Our in vitro experiments indeed show that the Ndc80 loop has major effects on the binding of Ndc80 ensembles to microtubules. To investigate this in dividing cells, and to dissect the contributions of the tail and the loop, and of Aurora B and Mps1, we performed the experiment now shown in **Figure 7** (formerly 7A).

Importantly, we agree with the reviewer that the 9A-tail mutant, by binding more strongly to microtubules, overrides the spindle assembly checkpoint. However, in the papers that we cite and that the reviewer refers to here, the 9A-tail was described as SAC-satisfying (Etemad *et al., 2015*) and SAC-silencing (Tauchman *et al*., 2015), also in cells with monopolar spindles. These terms were included in the title of these papers and we wanted to clarify that these mutants are rather SAC-overriding mutants.

In our experiments, the 9A mutant decreases checkpoint arrest under all conditions tested, and its effects in overriding the SAC are particularly strong when MPS1 is also partially inhibited (**Figure 7**). We thought it is important and worth reporting these findings as they enrich our understanding of the consequences of these mutations. In the revised version, we have also introduced textual changes to get this point across more clearly.

Referee #2:

In this work, Polley et al. investigate the role of the loop region of Ndc80 (a structured region at a break in an extended coiled-coil region) in the NDC80 complex's function. They demonstrate that the loop mediates microtubule-dependent homotypic interactions between NDC80 complexes in vitro, and that these interactions impact NDC80:microtubule binding stability. In addition, their experiments suggest that the Ndc80 loop has a role in proper kinetochore function and chromosome segregation in cells, consistent with prior work. Although the in vitro experiments suggest a novel and interesting role of the Ndc80 loop in NDC80 complex oligomerization and microtubule interactions, their in vitro and in vivo work seem largely disconnected and they fail to convincingly tie the oligomerization to NDC80 function in cells. In particular, there are multiple alternate explanations that could explain the cellular phenotypes that they have not excluded. At a minimum, the authors need to use extensive caution in interpreting these results, and should substantially rephrase their conclusions throughout the paper, although additional data could allow them to make stronger statements. In addition, although they mention prior work on the Ndc80 loop in their discussion section, they do not acknowledge these alternate possibilities throughout the text in a way that leads to a broad feeling of them overstating their conclusions.

We thank the reviewer for valuable comments and suggestions. In particular through the analysis of newly generated mutants, the connection between our *in vitro* and *in vivo* results is strengthened in the revised manuscript. Following the reviewer's advice, we have also rephrased and clarified our conclusions. We provide a point-by-point response below.

Major Points

1. The authors fail to link the Ndc80 oligomerization behavior to the cellular phenotype. It is clear from their work that the loop enables oligomerization in vitro and that the loop is necessary for proper chromosome congression in vivo, but there is no direct evidence that the congression defect is caused by a failure to oligomerize.

We appreciate the reviewer's concern. We would like to point out that each microtubule-binding site consists of a handful of Ndc80 complexes. The Ndc80 loop mutants localize normally. Imaging these attachment sites in vivo with the resolution required to address the reviewer's concern is currently not possible or, at least, has not yet been achieved by us or by others. Previously, the loop mutant had been proposed to fail to bind to the SKA complex, but in a previous study we showed that its ability to bind SKA in vitro is not impaired (please see also our answer to the next comment). We show here instead that Ndc80 oligomerization is impaired. This is currently the best explanation we have for the dramatic phenotype of Ndc80 loop mutants.

They also do not test some reasonable alternative hypotheses, including evaluating Ndc80 phosphorylation status (for example, using phospho-antibodies) or analyzing the localization of other outer kinetochore proteins, such as the SKA complex.

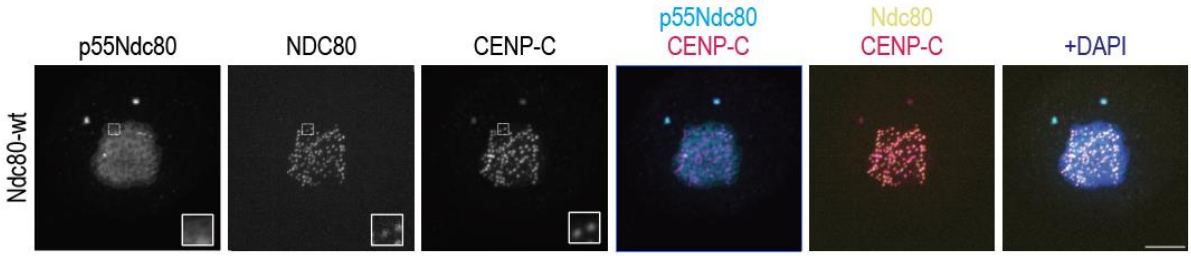
We have generated a novel antibody against the full-length SKA complex and characterized it for the revised version of our work (see **EV Figure 3D-G**), showing that it recognizes SKA3 in immunoblotting and the SKA complex in immunofluorescence.

Importantly, we find that when microtubules are depolymerized, SKA is recruited to kinetochores to similar levels in presence of wild type or loopless mutants (see **EV Figure 3H-I**). We also find that SKA accumulates on kinetochores in wild type cells at metaphase: a condition that the loop mutants never reaches (**EV Figure 3F-G**).This implies that basal levels of SKA can be recruited independently of the loop.

Such basal levels could not be detected at loopless kinetochores for the Astrin-SKAP complex, another microtubule-binder (see our comment to reviewer 3). However, as for the SKA complex, this does probably not reflect a direct involvement of the loop, but only an indirect consequence of the loop preventing attachment maturation. Thus, the ability of Ndc80 complex to form clusters may have downstream effects on the composition and chemistry of a kinetochore that are yet to be tested.

Loop-independent recruitment of SKA to kinetochores is consistent with previous *in vitro* results demonstrating that SKA forms a stable complex with loopless Ndc80 (Huis in 't Veld et al., eLife 2019). At first glance, however, this result may seem to contradict an earlier paper from the laboratory of Jakob Nilsson (Zhang et al., JCS 2012; "*The Ndc80 internal loop is required for recruitment of the Ska complex to establish end-on microtubule attachment to kinetochores"*). We suspect that the decreased SKA levels on the loop mutants reported by Zhang and colleagues can be explained by a lack of SKA recruitment to kinetochores of chromosomes that did not congress properly (consistent with Auckland et al., JCB 2017), which we suspect result from loop-mediated clustering upon microtubule attachment. Because loop mutations prevent biorientation, we compared SKA levels on wild type and mutant Ndc80 in absence of microtubules, i.e. under identical conditions. The levels of SKA were indistinguishable, as expected based on our previous biochemical analysis.

To address the relation between the Ndc80 loop and the phosphorylation of the Ndc80 tail, we investigated Ndc80 complexes with loop mutations and a non-phosphorylatable 9A-tail (**Figure 7**). During this revision, we also tried to analyse the phosphorylation status of the Ndc80 tail in complexes with mutations in the loop. Unfortunately, we encountered technical issues with this batch of the commercially available antibody (see image below) that were also confirmed by the antibody's vendor. We are therefore unable to answer this part of the question at this time.



2. The mutant that they chose to explore to analyze Ndc80 function in cells (M5) phenocopies ΔLoop in cells, but M5 trimers diffuse more similarly to wild-type than to ΔLoop. This suggests that its microtubule binding activity isn't that different from wild-type such that unstable microtubule binding caused by an oligomerization defect is not a common cause of ΔLoop and M5's phenotypes. This casts the authors' hypothesis into doubt.

We thank the reviewer for raising this concern. In order to address it, we generated two new structure-guided Ndc80 loop mutants for the revision of our paper. In the new mutants, called M13 and M14, either the hydrophobic or the acidic residues in the conserved DFEI stretch are mutated to Alanine. This stretch was previously covered with M5 (DF to AA) and M6 (EI to AA). To our excitement, the new mutants pointed out a crucial role for the exposed acidic residues D436 and E438 in both chromosome congression in cells and in oligomerization on a microtubule lattice *in vitro*. Mutation of the F437 and I439, contributing to the loop's hydrophobic cavity, resulted in Ndc80 complexes behaving like their wild-type equivalents.

These results provide insight into the predicted structure of the Ndc80 loop and connect our *in vitro* and *in vivo* results. We present these results prominently in our final figure (**Figure 8A-B**).

3. One way to explore this question further is to pursue additional mutants. They generated 11 mutants and observed several distinct phenotypes, but only characterized one. Their results suggest that the loop has at least two roles: one in oligomerization, and one in the SAC (although this may be related to microtubule binding indirectly). They could characterize those mutants in vitro to determine which ones have an oligomerization defects. If any of them have an oligomerization defect but don't cause a phenotype, it would suggest that the oligomerization defects does not cause the phenotype. If all

mutants that have an oligomerization defect also fail to congress their chromosomes, it would provide stronger support that oligomerization is important.

We thank the reviewer for this suggestion. We have now tested the behavior of several loop mutants (that either support of prevent normal chromosome congression) for their ability to oligomerize into clusters on microtubules. These data are presented alongside the characterization of the new mutants in **Figure 8B**.

This experiment was performed and analysed blindly, i.e. without providing the experimenter with information on the identity of the mutant. The experiment shows a very strong correlation between the clustering of Ndc80 complexes on microtubules in a reconstituted system and their ability to support chromosome congression. The only exception is the M6 mutant, whose effects are harder to interpret. The completely different behavior of the M13 and M14 mutants is striking and leads us to propose the acidic surface patch as an important interface in the clustering interaction we have identified.

We refer the reviewer to our response to the point 3 raised by Reviewer 1 above for additional experiments and a revised interpretation of the link between the SAC and the loop.

4. An alternative approach would be to force oligomerization in cells to rescue ΔLoop mutants. They attempted to do this with an antibody, but this approach has several caveats. First, IgG should only cause dimerization, which is likely not the case for the Ndc80 loop based on their in vitro results. While dimerization may be sufficient to rescue the microtubule-binding phenotype of a trimer in vitro, it might not be sufficient for a kinetochore with hundreds of NDC80 complexes. Second, antibodies frequently impair the functions of their targets in living systems (for example, Deluca et al. 2006 for the Ndc80 complex), as is likely the case here making their in vivo antibody experiments difficult to interpret. Finally, they don't show that these antibodies drive oligomerization in cells. Even if the antibody did not impair wild-type function, the authors would need to show that it actually oligomerizes NDC80 complexes in cells for these results to be interpretable. Because of these caveats, it would be preferable to attempt a similar experiment using an alternative approach. One possibility is oligomerizing tags (similar to Hara et al. 2022 BioRxiv), possibly on a different subunit of the complex that is less sensitive to tagging. However, I recognize that these experiments represent a substantial investment - another reason why using much more caution in their conclusions based on their existing data is important.

The reviewer rightly pointed out that the antibody results *in vivo* should be interpreted cautiously. We would like to refer to our response to the comments by Reviewer 1 for additional experiments and a revised interpretation of the link between the SAC and the loop. While we agree with the reviewer that it would be fantastic to control the oligomerization of Ndc80 complexes at a position near the microtubule-binding interface in cells, we note that this would be an immensely demanding engineering feat, in the absence of information on the relative orientation of the clustering complexes required for their correct function in vivo. In line with the results with the antibody in vitro and in vivo, we would also not consider a failure of such an experiment as evidence against the mechanism.

Minor Points

1. It could be worth showing that the fold of the NDC80 loop is dependent on a disulfide bond by adding a reducing agent.

We emphasized that the putative Cysteine bridge could significantly contribute to the stability of the loop’s switchback structure, but believe that an extended analysis of this putative cysteine bridge goes beyond the scope of this work.

2. Line 219: "in presence" should be "in the presence."

Corrected.

3. In Figure 3A, "bead restores with growing MT" is unclear. "Bead returns to center of laser" would be more clear.

Amended.

4. In figure 4E, the colors are very hard to distinguish for colorblind people. Red and Blue or Green and blue would be better.

We changed the color coding and thank the reviewer for pointing this out.

5. In figure 4E, it would be nice to put the amino acid numbers at the beginning and end of the sequences labeled.

Added.

6. In line 266, Figure 5B is referenced alone. 5B-D should be referenced.

Clarified the references to this figure.

7. In line 275, Figures 5D and Figure S6 are referenced. It should also reference figure 5B.

Clarified the references to **Figure 5** and **EV Figure 4** (formerly S6).

8. In Figure 5B, it needs to be made clear that the x axis is the position along the microtubule. It would also be nice to have axis labels on the first graph to make it clear that it's a kymograph.

Amended.

9. In Figure 6A, indicate which part of the sequence corresponds to which antibody.

Added.

10. It isn't clear how the section "Loop-mediated mitotic arrest involves multiple phospho-signaling pathways" relates to the rest of the story.

This section addresses synergistic contributions of the Ndc80 tail and theNdc80 loop and the role of Aurora B and Mps1 on kinetochore-microtubule attachment and checkpoint signalling. We hope that this is more clear following improvements of the text and now that the section on the putative effects of the loop on SAC signalling is dramatically shortened.

11. In Figure S8, add DAPI greyscale to show congression defects more clearly.

Changed.

12. In line 372, the authors state that "the region immediately preceding the loop [...] [is] important for SAC signaling." This is an overinterpretation. AB-849 could cause the phenotype by steric hindrance or by stabilizing an dysfunctional conformation of the complex even if the region it binds is not directly involved in SAC function.

We agree, as discussed above.

13. In line 386-388, the authors state that "the NDC80 loop promotes Ndc80-Ndc80 interactions that are crucial to generate force resistant attachments." This is also an overinterpretation. They show that the loop is essential for force-resistant attachment in vitro. They do not demonstrate that it is the oligomerization function of the loop that is essential for force-resistant attachments.

We have modified the sentence.

14. In lines 388-390, the authors state that "Our observations also suggest that interactions between adjacent Ndc80 complexes may signal the establishment of load-bearing kinetochore-microtubule attachments and silence the SAC." Nothing that they have shown supports this model.

Amended.

15. The word "nematic" (line 431) is obscure and distracting to the reader - use simpler language.

Nematic order was previously introduced as a relevant concept for Ndc80 by Roscioli and colleagues (Cell Reports, 2020). We revised this sentence for a better explanation and changed our summarizing cartoon to convey this concept (**Figure 8C**).

Referee #3:

This is an interesting study aiming to explore a new role of the Ndc80 loop in kinetochore-microtubule interactions. The authors raised a hypothesis that the loop-mediated Ndc80 clustering is critical for proper kinetochore-microtubule interactions. To test this hypothesis, the authors performed a series of biochemical, biophysical and cell-biological experiments. Overall, this is a good study and the results largely support the authors' conclusions. The findings could potentially contribute to the mechanistical understanding of how proper kinetochore-microtubule interactions are achieved. Several points need to be addressed before moving forward to the next step.

We thank the reviewer for the positive evaluation of our work and for their comments and suggestions.

1. The major point in this study is loop mediated Ndc80 clustering. The direct evidence for it is the results from Figure J and K. This clustering seems to be present only in the presence of microtubules. In the tube without microtubules, Ndc80 full-length and delta-L behave almost same in size-exclusion chromatography (suppl. Figure 3A). These observations suggest that binding of Ndc80 WT to microtubules might do "something" to allow Ndc80 clustering, whereas absence of loop fails to do so. I think that this possibility needs to be taken into account in the discussion.

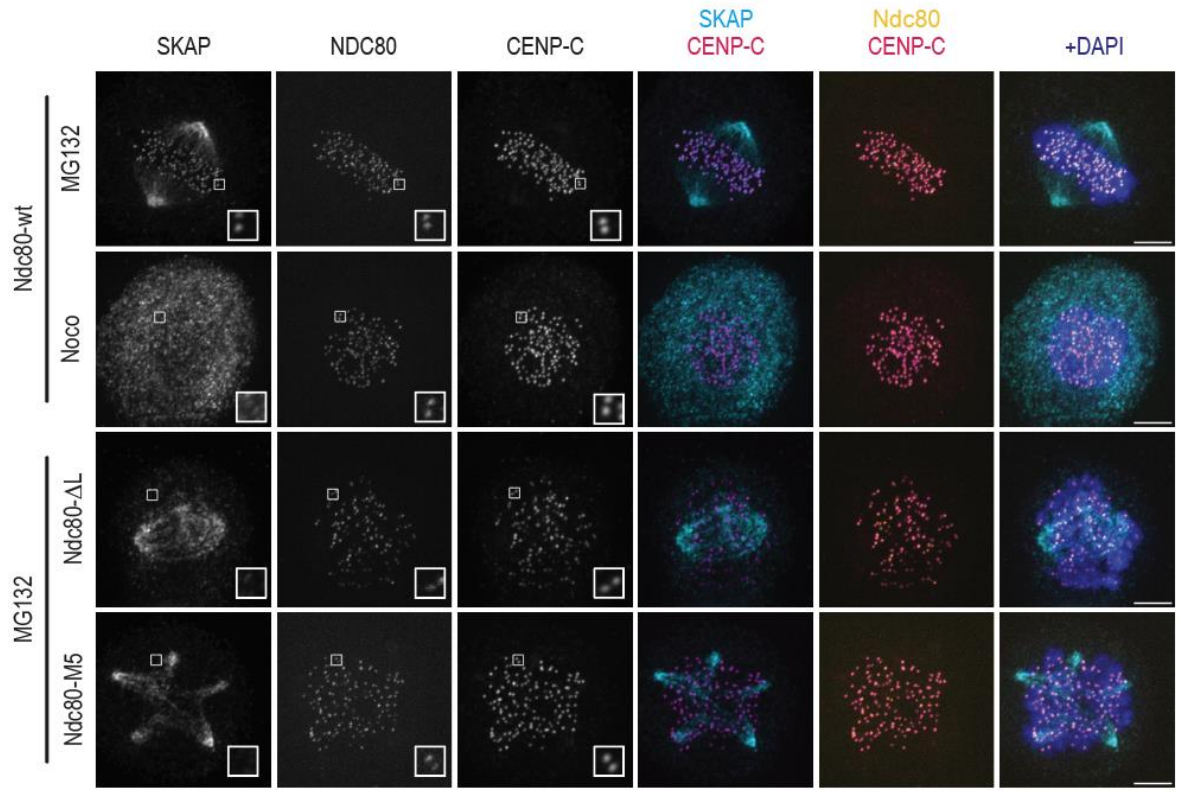
We fully agree with this interpretation: Ndc80 oligomerization requires both the loop and microtubules. This is a central point of our study and something that we tried to mention in our discussion already (second paragraph), but now also stress in the results section and in our new summarizing models (**Figure 8C-D**).

2. Although the idea of loop mediated Ndc80 clustering is interesting, how the loop does so is unclear. Several experiments could be performed to potentially provide a clue. Firstly, might the loop alone be oligomerized in vitro? Secondly, how are Ska and SKAP/Astrin kinetochore recruitment affected in M1, M2, M5, M6, and M10 mutants.

Following the reviewer's suggestion, we have produced a fragment of NDC80:NUF2 that encompasses the loop region. When immobilized on beads, this fragment did not engage in homotypic interactions and did not recruit full-length Ndc80 complexes (present in concentrations above 10 µM) (**Appendix Figure 3**). This negative result implies that the binding affinity for clustering must be low, a reasonable assumption if it must be triggered by microtubule binding on already closely localized Ndc80 complexes. We would also like to refer to the manuscript by Zahm, Jenni, and Harrison (bioRxiv, November 2022; Open Biology, March 2023) describing the stability and crystallization conditions of a similar fragment.

In the absence of microtubules, wild-type and loop mutants recruit similar levels of SKA. This is consistent with previous work (Huis in 't Veld et al., eLife 2019) showing that the loop is not required for the formation of a stable Ndc80:Ska complex *in vitro*. This result can be found in **EV Figure 3H-I**. We refer the reviewer to the detailed description of these new experiments provided to in our response to point 1 raised by Reviewer 2.

Astrin-SKAP complexes are only recruited to kinetochores after a stable binding to the microtubule ends is established (see for example Dunsch et al., JCB, 2011; Shrestha et al., Nat Comm., 2017). We do therefore not detect Astrin-SKAP in cells treated with the microtubule poison nocodazole or in cells with Ndc80 loop mutants (see below). The molecular basis for Astrin-SKAP recruitment to kinetochores remains unknown and a topic for future studies.



Minor points:

3. In Figure 5A, it seems that Ndc80-M5 is less recruited to kinetochores. Quantification is needed.

Wild-type and mutated electroporated Ndc80 complexes are on average recruited to kinetochores at similar levels. This data is shown in **EV Figure 3A**.

4. In Figure 8, the legend title should be "Synergistic contributions of the Ndc80.....". In Supple. Figure 7, the "B" is misplaced.

Amended.

Dr. Pim J. Huis in 't Veld
Max Planck Institute of Molecular Physiology
Mechanistic Cell Biology
Otto Hahn Strasse 11
Dortmund 44227
Germany

28th Apr 2023

Re: EMBOJ-2022-112504R
Stable kinetochore-microtubule attachment requires loop-dependent Ndc80-Ndc80 binding

Dear Pim,

Thank you again for submitting your revised manuscript for our consideration. Referees 1 and 2 have now assessed it once more, and are largely satisfied with the revisions and improvements of the manuscript. Nevertheless, referee 2 retains a few concerns regarding interpretation, which I would ask you to incorporate into the text in a final round of minor revision.

At this stage, there are also the following editorial points to still address:

- Please rename the Appendix Figures 1-5 into "Appendix Figure S1-5", both within the all occasions within Appendix file (ToC, legends, headers) and at all call-outs in the main text.

- At this stage, please also remove the Appendix Figure legends from the main text.

- Please enter a valid email address for co-author Helen Müschenborn into our system, as the last acknowledgement message sent by out office could not be delivered.

- Please reorganize the provided source data not according to data types, but according to figure. E.g. for figure 1, there should be on ZIP file containing all the image (scans, micrograms) and numerical (XLS) data for all panels in this figure. Numerical data from different figures/panels should not be combined into single XLSX files. Furthermore, please check the source data files for some of the images, as certain data types appear to open as blank/white files when downloaded onto my computer (e.g. Fig. 1F, 5B, 6B).

- Finally, please provide suggestions for a short 'blurb' text prefacing and summing up the study in two sentences (max. 250 characters), followed by 3-5 one-sentence 'bullet points' with brief factual statements of key results of the paper; they will form the basis of an editor-written 'Synopsis' accompanying the online version of the article. Please also upload a synopsis image, which can be used as a "visual title" for the synopsis section of your paper. The image should be in PNG or JPG format with the modest dimensions of (exactly) 550 wide x 300-600 pixels high.

I am therefore returning the manuscript to you for a final round of minor revision, to allow you to make these adjustments and upload all modified files. Once we will have received them, we should be ready to swiftly proceed with formal acceptance and production of the manuscript.

With kind regards,

Hartmut

Hartmut Vodermaier, PhD
Senior Editor, The EMBO Journal
h.vodermaier@embojournal.org

Further information is available in our Guide For Authors:
https://www.embopress.org/page/journal/14602075/authorguide

Use the link below to submit your revision:

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Referee #1:

The authors have addressed the comments satisfactorily. Especially the improved focus on the impressive biochemical and biophysical studies and the brief but transparent discussion on the findings with Ab849 on congression and the SAC greatly improved the flow of the paper. I have no further comments and support publication in the EMBO journal.

Referee #2:

In their revision, it is clear that Polley et al. invested substantial effort in addressing the prior comments from us and the other reviewers. However, even with these changes, the in vivo results remain inconclusive and the paper's conclusions are largely unchanged. The excellent and substantial in vitro analysis of the ΔLoop mutant warrants publication in EMBO Journal, but we recommend that the conclusions drawn from the in vivo work be toned down significantly to accurately reflect their results.

1. In their analysis of the correlation between the in vivo and in vitro phenotypes of the mutants (Figure 8), the authors find that ΔLoop, M5, and M13 all impair oligomerization in vitro and impair mitotic progression in cells. However, they also find that M6 impairs mitotic progression, but has no impact on oligomerization. That suggests that M6 affects another function of the loop that is important for mitotic progression. The mutations in M6 are immediately adjacent to those in M5 and overlap with those in M13. In light of that, there is a high likelihood that ΔLoop, M5, and M13 impact the same function as M6. This result casts additional doubt on the authors' conclusion that the cellular phenotype is due to impaired oligomerization. Given these doubts, it is essential that the authors are straightforward about the limited conclusions that they can draw from their in vivo work. I recommend that they attenuate their conclusions on the in vivo work throughout the paper, but also specifically highlight this point and alternative interpretations.
2. For the in vitro experiment in figure 6B, the authors used an anti-rabbit secondary to detect AB-849. Because the secondary IgG is also bivalent, this secondary antibody would be expected to further crosslink the trimers in due to dimerization of the AB-849, resulting in higher-order oligomers. It isn't clear whether these secondaries were also present in the experiments analyzed in 6C and 6D. If they are, then the crosslinking that the author induce in vitro may be quite different from the crosslinking that they induce in vivo. In either case, it would be helpful to clarify this point and comment on the likely behaviors.

Referee #1:

The authors have addressed the comments satisfactorily. Especially the improved focus on the impressive biochemical and biophysical studies and the brief but transparent discussion on the findings with Ab849 on congression and the SAC greatly improved the flow of the paper. I have no further comments and support publication in the EMBO journal.

We thank the reviewer for their help to improve the paper.

Referee #2:

In their revision, it is clear that Polley et al. invested substantial effort in addressing the prior comments from us and the other reviewers. However, even with these changes, the in vivo results remain inconclusive and the paper's conclusions are largely unchanged. The excellent and substantial in vitro analysis of the ΔLoop mutant warrants publication in EMBO Journal, but we recommend that the conclusions drawn from the in vivo work be toned down significantly to accurately reflect their results.

We thank the reviewer for the support and critical evaluation of our results and conclusions. Mutations in the loop have dramatic consequences for chromosome congression, despite being far away from the microtubule binding site. As clarified more thoroughly in our response to point 1, we have extended our analysis and demonstrated that the effects are recapitulated also in presence of more accurately targeted mutations than those we had described in the original manuscript. Nonetheless, we agree with the reviewer that we do not have direct proof that the mechanism we have identified is the sole source of the phenotype we observe. Thus, following the reviewer's advice, we now emphasise in the discussion that other explanations cannot be ruled out (lines 450-452). We hope that this accomodates the reviewer's concerns and would like to address points 1 and 2 specifically below.

Lines 450-452 of the discussion:
Old: *Collectively, our findings allow us to formulate a new hypothesis on the coordination of the molecular events that mark the process of bi-orientation. We surmise that ..*

New: *A challenge of any reconstituted system is that it may fall short of reproducing all the layers of regulation that contribute to a biological process. With this limitation in mind, we nevertheless formulate a hypothesis on the coordination of molecular events that mark the process of bi-orientation. Based on our findings, we surmise that ..*

1. In their analysis of the correlation between the in vivo and in vitro phenotypes of the mutants (Figure 8), the authors find that ΔLoop, M5, and M13 all impair oligomerization in vitro and impair mitotic progression in cells. However, they also find that M6 impairs mitotic progression, but has no impact on oligomerization. That suggests that M6 affects another function of the loop that is important for mitotic progression. The mutations in M6 are immediately adjacent to those in M5 and overlap with those in M13. In light of that, there is a high likelihood that ΔLoop, M5, and M13 impact the same function as M6. This result casts additional doubt on the authors' conclusion that the cellular phenotype is due to impaired oligomerization. Given these doubts, it is essential that the authors are straightforward about the limited conclusions that they can draw from their in vivo work. I recommend that they attenuate their conclusions on the in vivo work throughout the paper, but also specifically highlight this point and alternative interpretations.

The reviewer is right that M5 and M6 impact chromosome congression in a similar way, but affect clustering on the microtubules differently. Guided by the structural prediction, we therefore generated the mutants M13 and M14 and mutated either the solvent accessible residues (in M13) or the residues that point towards the loop's hydrophobic cavity (M14). The behaviour of these mutants indicated a role of the acidic patch in the loop on both Ndc80-Ndc80 interactions on microtubules in vitro, and on the ability to congress chromosomes in a dividing cell. We present these results prominently in figure 8 but could not repeat other experiments, such as the epistatic analysis with the Ndc80-tail, with the new mutants.

Following the reviewer's advice, we toned down the description of these results in the sentence below by ***removing*** two words. Lines 397-399:

*This experiment demonstrated the **strong** correlation between homotypic Ndc80-Ndc80 clustering on microtubules and stable kinetochore-microtubule interaction during mitosis and highlighted a **major** contribution of the loop's acidic patch formed by D436 and E438 (Figure 8A and Appendix Figure S5).*

2. For the in vitro experiment in figure 6B, the authors used an anti-rabbit secondary to detect AB-849. Because the secondary IgG is also bivalent, this secondary antibody would be expected to further crosslink the trimers in due to dimerization of the AB-849, resulting in higher-order oligomers. It isn't clear whether these secondaries were also present in the experiments analyzed in 6C and 6D. If they are, then the crosslinking that the author induce in vitro may be quite different from the crosslinking that they induce in vivo. In either case, it would be helpful to clarify this point and comment on the likely behaviors.

Primary and secondary antibodies were used together throughout Figure 6 because these data all originate from the same set of experiments. However, we only analysed single trimers for their diffusion (as described in Appendix Figure S4). While we do not know if/when the secondary antibody was bound and if/how it contributed, we do therefore know that the secondary antibody did not crosslink Ndc80 trimers.

We thank the reviewer for raising this point and have added the ***following clarification*** to the legend of Figure 6 in our revised manuscript:

*One-dimensional diffusion of full-length (blue), loopless (orange), and M5 (black) Ndc80 trimers in presence and absence of AB-849 and AB-850 **as described in panel B (see Appendix Figure S4 for more information)**. Traces of..*

Dr. Pim J. Huis in 't Veld
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Germany

8th May 2023

Re: EMBOJ-2022-112504R1
Stable kinetochore-microtubule attachment requires loop-dependent Ndc80-Ndc80 binding

Dear Pim,

Thank you for submitting your final revised manuscript for our consideration. I am pleased to inform you that we have now accepted it for publication in The EMBO Journal.

Your article will be processed for publication in The EMBO Journal by EMBO Press and Wiley, who will contact you with further information regarding production/publication procedures and license requirements. You will also be provided with page proofs after copy-editing and typesetting of main manuscript and expanded view figure files.

Should you be planning a Press Release on your article, please get in contact with embojournal@wiley.com as early as possible, in order to coordinate publication and release dates.

Thank you again for this contribution to The EMBO Journal and congratulations on a successful publication! Please consider us again in the future for your most exciting work.

With best regards,

Hartmut

Hartmut Vodermaier, PhD
Senior Editor, The EMBO Journal
h.vodermaier@embojournal.org

EMBO Press Author Checklist

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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
- 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

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 $\chi 2$ 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.
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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	

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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	still need to be double-checked and updated to latest standards

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Short novel DNA or RNA including primers, probes: provide the sequences.	Not Applicable	

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Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Yes	
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.	Not Applicable	

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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	

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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	
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If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

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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.	Yes	
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.	Yes	
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.	Yes	
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	

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	
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	

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 approva**l (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	
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**.	Not Applicable	