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A structural basis for kinetochore recruitment of the Ndc80 complex via two distinct receptors

Francesca Malvezzi, Gabriele Litos, Alexander Schleiffer, Alexander Heuck, Karl Mechtler, Tim Clausen and Stefan Westermann

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Transaction Report:

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

1st Editorial Decision 04 September 2012

Thank you for submitting your manuscript on the Ndc80-Cnn1 interface structure for consideration by The EMBO Journal. After some delay owed to the vacation season, we have now received the reports from three expert referees, copied for your information below. As you will see, these referees are generally supportive of eventual publication, although reviewers 2 and 3 do raise a number of points that may require additional experimentation for their clarification. Should you be able to satisfactorily address these points, we should be happy to consider a revised version of the manuscript further for publication. Please be reminded that it is our policy to allow a single round of major revision only, and that it will therefore be important to diligently and comprehensively answer to all the specific points raised at this stage in the process. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time, and it is our policy that competing manuscripts published during this period will have no negative impact on our final assessment of your revised study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider this work, and please do not hesitate to contact me in case you should have any additional question regarding this decision or the reports. I look forward to your revision.

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

This well-prepared paper presents the structure of a segment from Cnn1 (yeast equivalent of CENP-T) bound with Spc24/Spc25 -- the centromere proximal end of the Ndc80 complex. The clear demonstration of competition with the Mtw1/MIND complex and the detection of a related sequence in Dsn1 adds to the value of the new structure.

The paper is essentially ready for publication. I have only a few minor writing-style suggestions, of the kind that might normally be made by a copy editor. (As the authors are not native English speakers, I hope these suggestions will be useful.)

1. Overuse of "interestingly". Try to avoid such interjected adverbs ("strikingly" and "remarkably" are others, although I didn't find them in the MS), which are actually neither grammatically correct (see the paragraph on misuse of "hopefully" in Strunk & White, The Elements of Style, which all writers should have on their desk) nor helpful to the reader. (Strunk and White also inveigh against use of "interesting", pointing out that it is almost always better to omit it.)

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4. On p. 10: "For this purpose, we used a version of the Mtw1 complex harboring a deletion of the first 171 amino-acid residues of Dsn1 (Dsn1 N); this deletant is functional ...." (No comma after "complex"; change the relative clause to an independent one, because it otherwise "dangles" -- that it, the antecendent of "which" is not clear. In general, a relative clause should follow immediately the noun to which it relates -- in this case, "complex", which is the functional entity, not "deletion", which is of course not functional at all. I also changed the main clause from passive to active. Always try to use the active voice, unless that usage is ugly, forced, or confusing. Often it saves words, and it is almost always more forceful.)

5. On p. 11, delete "bioinformatically" (unnecessary and ugly).

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cannot refer to an entire sentence or an entire thought, and the true reference is not even "recent studies" but rather the results of those studies, i.e., the finding.

Referee #2 (Remarks to the Author):

Malvezzi and co-workers investigated the interaction of the Ndc80 complex, a crucial component of the outer kinetochore, with Cnn1/CENP-T, a conserved kinetochore protein of rather uncertain function. The authors report a structural characterization of a complex of the Spc24/25 subunits of the Ndc80 complex with a peptide encompassing their binding motif in Cnn1/CENP-T. The authors reveal the structural basis for the competitive interaction of Cnn1/CENP-T and the Mtw1/Mis12 complex with Spc24/25, and identify an Spc24/25-binding motif near the C-terminus of Dsn1, a subunit of the Mtw1/Mis12 complex. By applying an artificial plasmid segregation assay, the authors identify a second important motif in Cnn1, and propose that it might be important for high-affinity binding to Spc24/25. Finally, the authors demonstrate that Cnn1 is a substrate of Cdk1 and Mps1 kinases, and that such phosphorylation serves to inhibit the interaction of Cnn1 with the Ndc80 complex.

The work presented here is of very good quality and I have no specific serious technical comment. The manuscript is well written. My only concern regarding this paper is conceptual. In a recent paper in Nature Cell Biology (Schleiffer et al., 2012), the authors proposed that the Mtw1/Mis12 complex and Cnn1 independently recruit the Ndc80 complex, thus configuring two distinct pathways for Ndc80 recruitment. Here the authors find a dominant phenotype when expressing non-phosphorylatable mutants of Cnn1. Loss of Cnn1 does not have an overt phenotype and in fact it seems to bring about a growth advantage in cells expressing tagged versions of Nuf2 and Nnf1. All these observations leave the overall picture rather undefined, but if anything they seem to argue against the existence of two independent pathways of kinetochore recruitment of Ndc80. The authors do indeed back away from their previous model and in Figure 7 they argue that binding of Cnn1 and Mtw1/Mis12 complex might not necessarily occur concomitantly. In this rather confusing context, partly created by the authors' own previous claims, the overall significance of this contribution is rather uncertain. Besides the structural characterization of the Cnn1/Ndc80 interaction, which is per se interesting, the identification of an Ndc80-binding motif in Dsn1 is also interesting and important. On the other hand, the overall biological implications of this study remain obscure.

Referee #3 (Remarks to the Author):

This paper (Malvezzi et al.) from the Westermann lab describes the interaction between the outer kinetochore NDC80 complex and the CCAN component, Cnn1/CENP-T in budding yeast. This work is a nice extension of their earlier work, which demonstrated that Cnn1 directly binds the Spc24/25 globular domain of the NDC80 complex. In this earlier paper, the group demonstrated that the interaction relies on a small, unstructured domain in the N-terminus of Cnn1. They also demonstrated that NDC80 binding to Cnn1 and the Mtw1/Mis12 complex is competitive and concluded that the Mtw1 complex and Cnn1 both participate in recruiting NDC80 to kinetochores.

In the current Malvezzi et al. paper, the authors co-crystalize the globular domains of Spc24/25 with the small interacting peptide derived from Cnn1 and demonstrate that the Cnn1 peptide binds to a hydrophobic pocket in Spc24/25. They find that mutation of residues in the binding pocket of Spc24/25 is lethal to cells, whereas Cnn1 deletion is not. The authors then go on to show that this domain in Spc24/25 also binds the Mtw1 complex, and mutation of residues in the hydrophobic pocket prevents binding to both Cnn1 or Mtw1 complex. Using a plasmid segregation assay, in which they tether Cnn1 to a mini-chromosome and score its ability to properly segregate (by recruiting essential kinetochore components that link to MTs), they identify additional regions in the unstructured domain of Cnn1 required for its function (presumably in binding NDC80). The authors demonstrate that Cnn1 is phosphorylated by both Cdk1 and Mps1, and they carry out a series of experiments using either phospho-mimetic or phospho-mutant versions of Cnn1. Co-IP experiments reveal that Cnn1-16A (no phosphorylation by Mps1 or Cdk1) interacts more robustly with NDC80-C than wild-type Cnn1, and they conclude that phosphorylation of Cnn1 inhibits promotes Cnn1-NDC80 dissociation. An interesting finding in the paper is that expression of Cnn1-16A in cells

caused a temperature-sensitive growth defect. In contrast, Cnn1 deleted cells have no growth defects at all. This suggests that preventing dissociation of Cnn1-NDC80 is a problem for the cells.

The description of budding yeast Spc24/25 binding to a CENP-T peptide at the atomic scale should be of interest to the mitosis field and represents an advance in our understanding of kinetochore architecture. However, there are a few questions/comments that should be considered prior to publication (outlined below).

1. The authors find that the binding of the small Cnn1 peptide to Spc24/25 occurs at rather low affinity (3.5 uM). How does this compare to a larger fragment of Cnn1? Do the authors suppose that this is near the physiological binding strength? If so, is this much weaker than Mtw1-NDC080 binding?

2. In CO-IP experiments, Cnn1-16A pulls down more NDC80 than wild-type Cnn1, suggesting that the de-phosphorylated form of Cnn1 binds NDC80 more tightly than the phosphorylated form. They also show that Cnn1-16A expressing cells exhibit growth defects. What is the nature of this defect? Are kinetochores still attached to MTs? Is the presumption that phosphorylation of Cnn1 is required to dissociate NDC80-Cnn1 interaction, and if this can't happen, NDC80-C can no longer bind to Mtw1 (and this lack of binding is the cause of the defect)? Finally, does the Cnn1-16A mutant rescue a Mtw1 deletion? Meaning, if Mtw1C is not around, can Cnn1 that doesn't dissociate from NDC80 support KMT attachment and chromosome segregation (as suggested from the plasmid segregation assays)?

3. Related to the above point, the authors seem to pinpoint a key phosphorylation residue at S74. Is a single phospho-mutant (S74A) sufficient to prevent dissociation of NDC80 from Cnn1 (and result in growth defects)?

1st Revision - authors' response 29 November 2012

Many thanks for the positive response to our manuscript “A structural basis for kinetochore recruitment of the Ndc80 complex via two distinct centromere receptors” by Malvezzi et al. We have addressed the remaining suggestions and questions of the reviewers by additional experimentation. We have furthermore added a particularly revealing experiment: We show that the lethal truncation of the Mtw1 complex subunit Dsn1 (which is defective in Ndc80 recruitment) is rescued in vivo by fusing the short Ndc80-binding motif from Cnn1 to the carboxyterminus. This demonstrates that the Ndc80 binding motif that we structurally characterize in our manuscript is functional in the context of a different molecule and thus reveals the modular nature of Ndc80 binding to the kinetochore. We hope that this revised manuscript is now acceptable for publication.

Please find below our point-by-point response to the Reviewers, our answers in bold print.

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We thank the reviewer for these helpful suggestions and we have incorporated them into the revised manuscript.

Reviewer 2

All these observations leave the overall picture rather undefined, but if anything they seem to argue against the existence of two independent pathways of kinetochore recruitment of Ndc80. The authors do indeed back away from their previous model and in Figure 7 they argue that binding of Cnn1 and Mtw1/Mis12 complex might not
necessarily occur concomitantly. In this rather confusing context, partly created by the authors' own previous claims, the overall significance of this contribution is rather uncertain. Besides the structural characterization of the Cnn1/Ndc80 interaction, which is per se interesting, the

identification of an Ndc80-binding motif in Dsn1 is also interesting and important. On the other hand, the overall biological implications of this study remain obscure.

We have tried to clarify these points in the revised manuscript. We did not intend to stress the idea that Ndc80 binding to Mtw1-C and Cnn1 needs to happen sequentially in a way that all Ndc80-Mtw1 linkages are converted into Ndc80-Cnn1 linkages (We have altered the model in Figure 8B accordingly). We do not have experiments that would lend strong support to this model. Our experiments do however show that unmodified Cnn1 is a high-affinity binding partner for Ndc80-C. A biochemical consequence of Cnn1 phosphorylation, which occurs early in the cell cycle, is a reduction of the affinity for Ndc80, which can help to promote the formation of Mtw1-Ndc80 links by avoiding competition. Our experiments show that the two Ndc80 recruitment pathways in budding yeast are clearly not functionally equivalent: Cnn1 is a non-essential protein and various genetic attempts to bypass the essential Ndc80-Mtw1 link with non-phosphorylatable Cnn1 or Cnn1 fusions have failed (see response to reviewer 3). We would also not like to stress the idea of "independent" Ndc80 recruitment pathways: Cnn1 recruitment to kinetochores is dependent on other CCAN subunits and thus ultimately on CENP-A and CENP-C.

We have tried to focus the manuscript on the clear findings of our study: The description of the Ndc80-Cnn1 interaction site in atomic detail, the discovery of a key interface between Mtw1-C and Ndc80 and the demonstration that the short Ndc80 binding motif from Cnn1 is modular and can be functionally switched into the Mtw1-C.

Reviewer 3:
1. The authors find that the binding of the small Cnn1 peptide to Spc24/25 occurs at rather low affinity (3.5 uM). How does this compare to a larger fragment of Cnn1? Do the authors suppose that this is near the physiological binding strength? If so, is this much weaker than Mtw1-NDC080 binding?

We have now included ITC data for the interaction between Spc24/25 and the full Cnn1 N-terminus (lacking only the histone-fold) (Figure 5C). The low dissociation constant (16 nM) indicates that this is a high-affinity interaction, similar or even stronger than the reported Ndc80-Mtw1-C association. This shows that full-length, non-phosphorylated Cnn1 can effectively compete with Mtw1-C for Ndc80 binding.

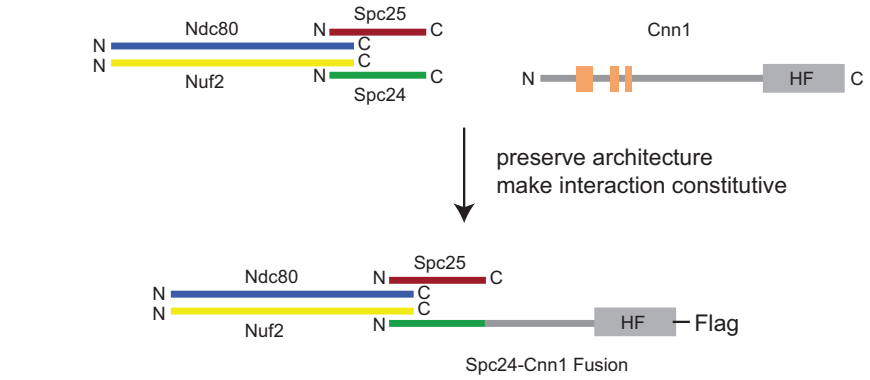
2. In CO-IP experiments, Cnn1-16A pulls down more NDC80 than wild-type Cnn1, suggesting that the de-phosphorylated form of Cnn1 binds NDC80 more tightly than the phosphorylated form. They also show that Cnn1-16A expressing cells exhibit growth defects. What is the nature of this defect? Are kinetochores still attached to MTs? Is the presumption that phosphorylation of Cnn1 is required to dissociate NDC80-Cnn1 interaction, and if this can't happen, NDC80-C can no longer bind to Mtw1 (and this lack of binding is the cause of the defect)?

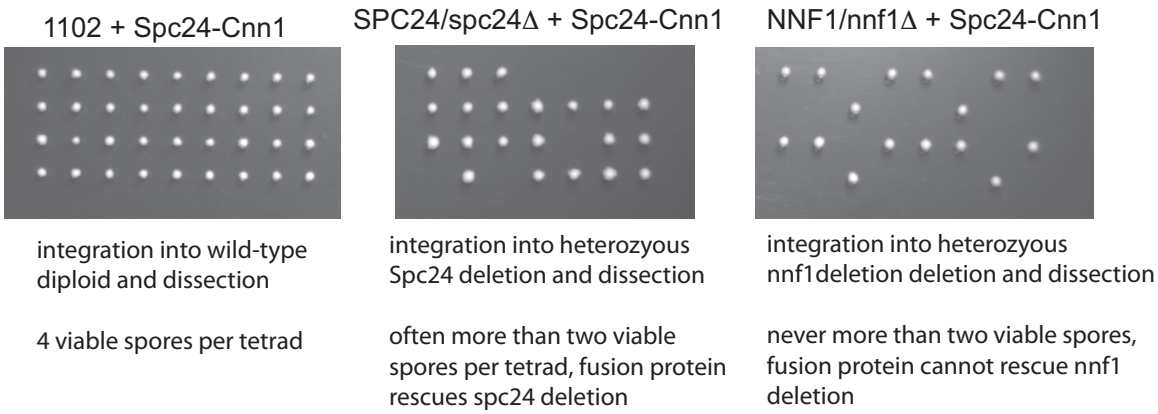
We have characterized the growth defect of Cnn1-16A further. At 37°C Nuf2-myc Nnf1-HA cells displayed a delay in cell cycle progression which was partially eliminated in combination with the Cnn1-16A mutant (Figure 7D). The Nuf2-myc Nnf1-HA Cnn1-16A cells did not display an overt arrest phenotype, but indirect immunofluorescence against tubulin and DNA indicated pleiotropic mitotic defects, including unequal segregation and short spindles with "cut" DNA (Figure S6). There were no indications that chromosomes were unattached from the spindle in these cells. We feel this is as far as we can go with the characterization of this phenotype, because the defects depend on the Nuf2-myc Nnf1-HA background. The molecular nature of the specific defect in these cells is difficult to address. Our finding that a single amino acid substitution in Cnn1 (S74D) is sufficient to improve growth of these cells is consistent with the idea that Nuf2-myc Nnf1-HA cells have problems in recruiting the Ndc80 complex effectively.
Our interpretation is that phosphorylation of Cnn1 decreases the affinity for Ndc80 and thus supports formation of the essential Mtw1-Ndc80 linkage.

Finally, does the Cnn1-16A mutant rescue a Mtw1 deletion? Meaning, if Mtw1C is not around, can Cnn1 that doesn't dissociate from NDC80 support KMT attachment and chromosome segregation (as suggested from the plasmid segregation assays)?

We have performed the suggested experiment. So far, in a plasmid shuffle assay we did not see that expression of Cnn1-16A would overcome the loss of Dsn1.

We have also performed a variation of this experiment by fusing the C-terminus of Spc24 to the N-terminus of Cnn1 with the idea that this operation eliminates all cell cycle control of the interaction. This fusion protein was functional as it could rescue an spc24 deletion, but it failed to rescue an nnf1 deletion (see Figure below). In addition we have fused the lethal Spc24 L160D mutant to the N-terminus of Cnn1, creating a situation where cells still have a functional Mtw1 complex. In genetic crosses we failed to recover spores that contained the Spc24L160D-Cnn1 fusion as the sole source of Spc24. Thus, we have not been able to bypass the essential connection between Mtw1-C and Ndc80-C in budding yeast by fusing Ndc80 to Cnn1.





3. Related to the above point, the authors seem to pinpoint a key phosphorylation residue at S74. Is a single phospho-mutant (S74A) sufficient to prevent dissociation of NDC80 from Cnn1 (and result in growth defects)?

We have tested the contribution of S74 phosphorylation in the revised manuscript more closely. The phospho-mimicking mutation S74D is sufficient to disrupt the Ndc80-Cnn1 interaction both in vitro (Figure 6G) and in vivo (Figure 6H). The mutant suppresses the growth defect of the Nuf2-myc Nnf1-HA strain (Figure 6I). The S74A single mutant increases the amount of Cnn1 co-immunoprecipitated with Nuf2 from log phase cells (supporting the notion that this residue is indeed

phosphorylated in cells). It slightly aggravates the growth defect of Nuf2-myc Nnf1-HA, but this effect is not as strong as in the 16A mutant. Overall this indicates that S74 contributes to the phosphoregulation in vivo, but the more extensive phosphorylation of the N-tail is required for the full regulation of the protein.

Acceptance letter 21 December 2012

Thank you for submitting your revised manuscript for our consideration. It has now been reviewed once more by the original referee 3, and I am happy to inform you that this referee is fully satisfied by the revisions and has no further concerns. We have therefore decided to accept the manuscript for publication in The EMBO Journal!

Thank you again for your contribution to our journal and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

