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CENP-T provides a structural platform for outer kinetochore assembly

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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 CENP-T/Spc24-25 interactions and their phospho-regulation for consideration by The EMBO Journal, and please excuse the delay in its evaluation due to the summer vacation period. Three expert referees have now assessed it, and their comments are copied below. Although these reports acknowledge potential importance and interest in your findings and conclusions, they also raise various major concerns that in our view currently preclude publication of the manuscript, at least in its present form. In particular referees 2 and 3 mention a number of both internal contradictions as well as discrepancies with earlier published work and interpretations, which would need to be decisively clarified. Furthermore, there are well-taken criticisms from both referees 1 and 2 regarding the sole reliance in phosphomimetic mutations, rather than the use of phosphorylated peptides in at least some key experiments; and related to that the insufficient dissection of specific roles of individual phosphorylation sites T72 and S88. Finally, there are also shared concerns regarding presentation, interpretation and writing.

I realize that decisively addressing these various points may require significant amounts of further time and (experimental) efforts, and would therefore understand if you were to rather seek rapid publication without major changes in another journal. Nevertheless, I do feel that revisions along the lines suggested by the referees could make the manuscript a much more conclusive and compelling candidate for an EMBO Journal paper, which could then also nicely complement the co-submitted manuscript; and I would therefore like to give you an opportunity to address the referees' concerns in the form of a revised manuscript. In this special situation, it may be helpful if you could write back to me with an outline of how you would like to address the most important referee concerns, so that we could discuss possibilities for further proceedings.

Please be reminded that competing manuscripts published here or elsewhere while you paper is

formally under EMBO J revision will have no negative impact on our final assessment of your revised study, but that we may in the present case not be in a position to offer revision time extensions beyond the three months standard revision time we generally allow.

Thank you for the opportunity to consider this work publication, and please do not hesitate to get back to me should you require further clarification regarding this decision or your revision.

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

This paper is an account of the structure of a complex of Spc24/Spc25 with a bound peptide from the N-proximal part of CENP-T. The structure is informative, and it illustrates that the interaction is similar in character to many that involve a short recognition element on one partner and a folded domain on the other (e.g., SH3, domains, PDZ domains, Kix/Kid, Bub3, etc.). In all such cases, the short recognition element is unstructured when not bound (these elements are generally too small to have any structure on their own) and acquires structure when bound.

Two features of the association attract the authors' attention: (1) the requirement that the peptide have negative charge at Cdk phosphorylation sites (they use a so-called phosphomimetic peptide, with Asp instead of phospho-Ser or phospho-Thr); (2) the absence of apparent contact between one of these negatively charged positions and Spc25 and instead a salt bridge between the Asp in question and an Arg two residues further along in the CENP-T fragment.

The paper includes some mutational data that connect the structure to functional information on Ndc80 recruitment to kinetochore and show that this contact does indeed have a role in Ndc80-kinetochore interaction. It further shows that binding the CENP-T fragment excludes interaction with the other link between Ndc80 and the inner kinetochore, through Dsn1 (and thence to CENP-C).

The authors have modified the MS in response to many of the criticisms I had when I reviewed it for another journal. It is still wordier and more convoluted than it needs to be, but aside from urging the authors to try to eliminate the relatively frequent repetition when they prepare the final version for publication, I will not try to make further suggestions. (One consistent incorrect English usage is "in total", as in the last sentence of the abstract and one or two other places. The authors mean "in summary" or "in conclusion", but the phrase is in any case unnecessary, so they should just eliminate it altogether. There are many other non-idiomatic or slightly incorrect uses of English, so EMBO J should make sure that a good copy editor has a careful look.)

I have two questions, but I do NOT believe getting the answers should hold up publication at this stage. Why didn't the authors use a synthetic peptide with a phosphoThr at 72 instead of an Asp, to eliminate any remaining doubts about whether the Asp is really an accurate mimetic (after all, it only has one negative charge, not two, so it can't really be "phosphomimetic")? And why didn't they look at the singly modified peptide, once the structure showed them which one seems to matter more? These experiments, when done, could improve the precision of the conclusions.

Referee #2 (Remarks to the Author):

The manuscript by Nishino and co-workers investigates the direct interaction of CENP-T, a recently discovered kinetochore protein, with the Ndc80 complex. The authors demonstrate in vitro a dependency of the interaction on phosphorylation of two sites on CENP-T. The authors report a crystal structure of the Spc24/25 region of Ndc80 bound to a phospho-mimetic peptide derived from the CENP-T sequence. The authors argue that their data support the existence of two distinct pathway of kinetochore recruitment of Ndc80, a CENP-T-dependent pathway and a Mis12-dependent pathway.

There are many merits in this manuscript, and I am confident that it will eventually make for a useful addition to the kinetochore field. However, there are also several discrepancies and inconsistencies with previously published data (including data from the authors' laboratories) that make this manuscript unsuitable for publication in its present form. The following specific points are especially cogent:

Abstract: on referring to the interaction of CENP-T and of the Mis12 complex to Ndc80 the authors write "...we find that these interactions required different residues in Spc24/24 and are mutually exclusive, supporting a model in which two distinct pathways target the Ndc80 complex to the kinetochore". Because these interactions are competitive, at least some of the residues involved must be the same, unless competition reflected an allosteric mechanism. This is rather unlikely, because structural analysis seems to indicate that the Spc24/25 moiety is remarkably stable (e.g. no structural change between the phosphor-mimetic peptide-bound form and the form devoid of peptide).

Page 4: "However, based on structural predictions, the N-terminus of CENP-T is unstructured and it is unclear how it would associated with the Ndc80 complex". There are dozens of examples of interactions of unstructured segments of proteins with folded domains, and therefore the sentence should not start with "however". The authors can write that it is unclear how "it associates" with the Ndc80 complex rather than "would associate". Also, please note that "N-terminus" literally refers to the amino group of the N-terminal residue. The authors are referring to the N-terminal region of CENP-T, rather than its N-terminus. The authors should also correct other instances in the text.

Pages 7-8 and Figure 2: The authors have previously reported a tight interaction of unphosphorylated CENP-T with Ndc80-Bonsai. In this report, however, they claim that Spc24-25 is unable to bind the unphosphorylated form of CENP-T and that phosphorylation is required for a high-affinity interaction. The authors should clarify this confusing discrepancy, and in particular if the unphosphorylated CENP-T peptide (or MBP fusion protein) that does not bind Spc24-25 is instead able to bind Ndc80-Bonsai. If not, one would conclude that additional segments of CENP-T are involved in binding. Which ones? If yes, on the other hand, one would conclude then additional parts of Ndc80 (presumably present in Bonsai) are involved. In either case, clarification is required.

Also, it is inadequate to omit the binding data for the CENP-T phosphopeptide, which after all is proposed to be the relevant binding species, just to limit oneself to phosphor-mimetic mutants. How does the binding affinity of a phosphopeptide compare with the binding affinity of phospho-mimetic mutants? This is particularly important in view of the fact that the authors report a structure of the phosphor-mimetic mutant rather than a phosphorylated species.

Figure 3: It would be helpful if the authors used sufficiently different colors to represent the Spc24 and Spc25 subunits.

Page 10 and Figure 4: After reading the paper, it is unclear if and to what extent phosphorylation of S88 contributes to the overall binding affinity of the CENP-T-Ndc80 interaction. Because a phospho-mimetic version of this residue is used throughout the manuscript, it is important that the authors clarify if phosphorylation of this residue is relevant and discuss it in the context of the structure. In the present version of the manuscript, the function of this residue is (rather surprisingly) completely neglected.

Page 10 and Figure 5: The authors report that the binding of CENP-T and Mis12 to Ndc80 is mutually exclusive. This is surprising in light of data contained in one of the authors' previous papers (Gascoigne et al., Cell 2011, Figure 4) that CENP-T binds Mis12. Here such interaction seems to disappear and is replaced by a competitive interaction with Ndc80. The authors should clarify this apparent discrepancy and clearly refer to it in the text.

Page 11: "...to provide a distinct pathway for outer kinetochore assembly (Hori et al., 2008; Gascoigne et al., 2011)." Neither citation is correct. The demonstration was first provided by the Przewloka et al. 2011 paper in Current Biology (incidentally, these authors showed that the CENP-C-Mis12 interaction is the crucial interaction in Drosophila, where CENP-T is probably absent). Second, there is no evidence that the pathways are distinct.

Page 12: "In total, we conclude that the Mis12 complex binds to the Spc24/25 complex using

distinct interaction residues from CENP-T, and the binding of the CENP-T N-terminus and the Mis12 complex to the Spc24/25 is mutually exclusive likely due to steric occlusion of this interaction surface". This formulation is slightly confusing, but once again, if the interaction is competitive, is it because the binding sites of Spc24/25 for Mis12 and CENP-T partly overlap? Or not?

Page 12-13 and Figure 6: The experiment is inconclusive. There is no quantitative understanding for how mutations on the CENP-T binding interface of Spc24/25 affect Mis12 binding. Once again, if the binding of CENP-T and Mis12 to Spc24/25 is competitive, most likely the mutations on Ndc80 affect Mis12 binding too, so that the observed reduction of Ndc80 at kinetochores might not simply reflect loss of CENP-T binding. To be rigorous, the authors should measure the binding affinity of Mis12 to Bonsai and mutant Bonsai and show that they are identical. Only then they will be able to support their claim. Co-elution in gel filtration experiments is not sufficient, because there might be sufficient residual binding affinity of Mis12 to the Ndc80 complex, and yet this might not be sufficient in cells.

Discussion: There are other instances of main chain stabilization by phosphorylation (e.g. Nature 430, 223-226 (8 July 2004) Recognition of RNA polymerase II carboxy-terminal domain by 3'-RNA-processing factors, Anton Meinhart & Patrick Cramer)

Referee #3 (Remarks to the Author):

In the manuscript, "CENP-T provides a structural platform for outer kinetochore assembly," the authors describe the interaction between Spc24/25 of the NDC80 complex and the inner kinetochore component CENP-T. This study extends earlier work by the authors that reported an interaction between these two molecules, and here they more finely define the interaction domains and analyze relationships between the NDC80 complex, the Mis12 complex, and CENP-T. Specifically, the authors map a small domain of CENP-T that is sufficient to bind the globular domains of Spc24 and Spc25. It was previously reported that the CENP-T/NDC80 interaction is dependent on phosphorylation, and the authors here confirm that finding by demonstrating that a phospho-mimetic CENP-T fragment binds Spc24/25 tightly, while the unmodified fragment does not. They go on to crystallize a region of Spc24/25 with the phospho-mimetic CENPT peptide and demonstrate that CENP-T binds to a hydrophobic pocket in Spc24/25. This binding interface is relevant in vivo, since they demonstrate that mutation of residues in this hyrdrophobic pocket inhibit wild-type levels of NDC80 recruitment to kinetochores in cells. Interestingly, they show that phosphorylation of the CENPT fragment does not facilitate direct contact with Spc24/25, rather it facilitates the formation of a salt bridge to allow residues in the hydrophobic pocket of 24/25 to bind to CENP-T. The authors go on to demonstrate that mutation of residues in the binding pocket does not affect NDC80 complex binding to Mis12 complex, suggesting Mis12C binds to NDC80 through a different motif; however, the NDC80 complex was unable to bind Mis12C and the CENP-T fragment simultaneously. They suggest that the mutually exclusive binding is due to steric hindrance.

The description of Spc24/25 binding to CENP-T at the atomic scale is a significant advance, and mitosis aficionados will find this work of interest. Specific comments and questions regarding the manuscript are outlined below.

1. What level of NDC80 is recruited to kinetochores in the absence of a functional CENP-T recruitment site? The answer to this question is not clear: throughout the paper, there are contradicting images, quantitative values, and estimations. The level of NDC80 at kinetochores under each of the experimental conditions should be quantitated. For example, in Figure 1C, the authors report that only 3.4% of NDC80 is left at kinetochores after expressing a CENP-T del69-90 fragment. However, later in the paper (Figure 6, Supp Figure 5), the authors claim that preventing CENP-T-based recruitment only reduces NDC80 at kinetochores by an estimated 50%. Where does this estimation come from? Why are these values so different?

2. Related to this point, if there are two parallel pathways for NDC80 recruitment, why don't we see NDC80 at kinetochores in Figure 1C (presumably the CENPT pathway is inactive, but shouldn't Mis12C compensate?)

3. Again, in Figure 6A, the authors present data suggesting that cells expressing the Spc mutant (that cannot bind CENP-T) have some residual levels of NDC80 at kinetochores. How much is there? In the images it looks like very little, but in Figure 6C, this level is clearly enough to support survival of the cells.

4. Related to the above point: in the graph in Figure 6, the difference between survival in tet on and tet off cells looks very minor. Is this significant?

5. Why is a CENP-T deletion lethal (Figure 1E), but cells expressing the Spc mutant (that cannot bind CENP-T) survive and proliferate (Figure 6C)? Do the authors suppose that CENP-T has some additional function not related to recruitment of NDC80 to kinetochores?

6. It is unclear how the authors envision the recruitment of NDC80 by two parallel pathways. Based on the proposed phospho -regulation of CENP-T/NDC80 binding, in early mitosis is CENP-T the major receptor for NDC80? As mitosis progresses and CENP-T is dephosphorylated, does NDC80 switch to Mis12 binding? Have the authors tested if a phospho-mimetic mutant of CENP-T affects mitotic progression (to prevent "release" of NDC80 from CENPT)? Some discussion of how these two parallel pathways may function in mitosis would be helpful.

1st Revision - authors' response 29 November 2012

Response to Reviewers Nishino et al. (EMBOJ-2012-82857)

Reviewer #1

1- The authors have modified the MS in response to many of the criticisms I had when I reviewed it for another journal. It is still wordier and more convoluted than it needs to be, but aside from urging the authors to try to eliminate the relatively frequent repetition when they prepare the final version for publication, I will not try to make further suggestions. (One consistent incorrect English usage is "in total", as in the last sentence of the abstract and one or two other places. The authors mean "in summary" or "in conclusion", but the phrase is in any case unnecessary, so they should just eliminate it altogether. There are many other non-idiomatic or slightly incorrect uses of English, so EMBO J should make sure that a good copy editor has a careful look.)

We appreciate the constructive comments and suggestions that we have received from this Reviewer both at EMBO J and at a previous journal. These comments have been very useful for us improving this paper, and we have continued to edit the paper and conduct experiments accordingly.

For the writing, in this revised version we tried to eliminate repetition and removed “in total” in the last sentence of the abstract and other places. We have also edited the text for proper English usage and wording.

2- I have two questions, but I do NOT believe getting the answers should hold up publication at this stage. Why didn't the authors use a synthetic peptide with a phosphoThr at 72 instead of an Asp, to eliminate any remaining doubts about whether the Asp is really an accurate mimetic (after all, it only has one negative charge, not two, so it can't really be "phosphomimetic")? And why didn't they look at the singly modified peptide, once the structure showed them which one seems to matter more? These experiments, when done, could improve the precision of the conclusions.

For the revised paper, we prepared synthetic phospho-peptides of CENP-T (one peptide contains phospho-Thr at 72 and another one contains both phospho-Thr at 72 and phospho-Ser at 88) and confirmed that these peptides bind to the Spc24/25 complex. We also measured the K_D for these binding interactions by ITC. These new data are included in Figure 2 of the revised version.

In these experiments, we found that a singly modified peptide (phospho-Thr 72) strongly increases binding to the Spc24/25 complex, and that this is further increased in the doubly modified peptide (T72p and S88p). Although we propose that phosphorylation of Thr72 is the most critical for facilitating this binding interaction based on our structural analysis, Ser88p may also contribute to this binding. For this reason, we used doubly phospho-mimetic CENP-T (T72D and S88D) for the other assays in this paper.

Reviewer #2

1- Abstract: on referring to the interaction of CENP-T and of the Mis12 complex to Ndc80 the authors write "...we find that these interactions required different residues in Spc24/24 and are mutually exclusive, supporting a model in which two distinct pathways target the Ndc80 complex to the kinetochore". Because these interactions are competitive, at least some of the residues involved must be the same, unless competition reflected an allosteric mechanism. This is rather unlikely, because structural analysis seems to indicate that the Spc24/25 moiety is remarkably stable (e.g. no structural change between the phosphor-mimetic peptide-bound form and the form devoid of peptide).

We have now measured the binding affinity of the mutant Spc24/25 complex with the Mis12 complex by ITC. The mutant Spc24/25 complex (I149A_L154A for Spc25) did not strongly associate with phospho-mimetic CENP-T, and also displays reduced binding affinity with the Mis12 complex. Whereas K_D for interaction of the wild-type Spc24/25 complex with the Mis12 complex was 18.9 nM, K_D for that of the mutant Spc24/25 with the Mis12 complex was 291 nM. Although the mutant Spc24/25 complex still binds to the Mis12 complex, we conclude that the binding sites of Spc24/25 for Mis12 and CENP-T partially overlap. We believe that this additional data explain the competitive binding of CENP-T and the Mis12 complex with the Spc24/25 complex. In the revised version, we present these data and have modified the abstract and main text.

2- Page 4: "However, based on structural predictions, the N-terminus of CENP-T is unstructured and it is unclear how it would associated with the Ndc80 complex". There are dozens of examples of interactions of unstructured segments of proteins with folded domains, and therefore the sentence should not start with "however". The authors can write that it is unclear how "it associates" with the Ndc80 complex rather than "would associate". Also, please note that "N-terminus" literally refers to the amino group of the N-terminal residue. The authors are referring to the N-terminal region of CENP-T, rather than its N-terminus. The authors should also correct other instances in the text.

We agree with these comments and have modified text on page 4 in line with this suggestion.

3- Pages 7-8 and Figure 2: The authors have previously reported a tight interaction of unphosphorylated CENP-T with Ndc80-Bonsai. In this report, however, they claim that Spc24-25 is unable to bind the unphosphorylated form of CENP-T and that phosphorylation is required for a high-affinity interaction. The authors should clarify this confusing discrepancy, and in particular if the unphosphorylated CENP-T peptide (or MBP fusion protein) that does not bind Spc24-25 is instead able to bind Ndc80-Bonsai. If not, one would conclude that additional segments of CENP-T are involved in binding. Which ones? If yes, on the other hand, one would conclude then additional parts of Ndc80 (presumably present in Bonsai) are involved. In either case, clarification is required.

We previously used human CENP-T to test its interaction with the Ndc80 complex. For this revised version, we examined whether MBP-fusions with unphosphorylated human CENP-T bind to Spc24/25. Unlike unphosphorylated chicken CENP-T, unphosphorylated human CENP-T associated with the Spc24/25 complex by gel filtration. Phosphorylation of CENP-T further enhances this association. We also measured the K_D for this interaction by ITC (645 nM). In revised version, these data are included in Figure S1.

4- Also, it is inadequate to omit the binding data for the CENP-T phosphopeptide, which after all is proposed to be the relevant binding species, just to limit oneself to phosphor-mimetic mutants. How does the binding affinity of a phosphopeptide compare with the binding

affinity of phospho-mimetic mutants? This is particularly important in view of the fact that the authors report a structure of the phosphor-mimetic mutant rather than a phosphorylated species.

As described above in response to comments from Reviewer #1, we have added new binding data using the CENP-T phospho-peptides to Figure 2 of the revised version.

5- *Figure 3: It would be helpful if the authors used sufficiently different colors to represent the Spc24 and Spc25 subunits.*

Based on this suggestion, we have modified Figure 3 to use different colours.

6- *Page 10 and Figure 4: After reading the paper, it is unclear if and to what extent phosphorylation of S88 contributes to the overall binding affinity of the CENP-T-Ndc80 interaction. Because a phospho-mimetic version of this residue is used throughout the manuscript, it is important that the authors clarify if phosphorylation of this residue is relevant and discuss it in the context of the structure. In the present version of the manuscript, the function of this residue is (rather surprisingly) completely neglected.*

Based on this comment and a similar suggestion from Reviewer #1, we prepared both a single phosphorylated CENP-T peptide (phospho-Thr72) and a CENP-T mutant in which only T72 is replaced with Asp. We found that single phosphorylation or singly phospho-mimetic CENP-T is sufficient to enhance binding to Spc24/25. In addition, our structural study revealed that T72D makes a major contribution to the binding of CENP-T to Spc24/25. However, the binding affinity of the double phospho-peptide (T72p and S88p) to Spc24/25 further increases this interaction relative to singly phosphorylated CENP-T (see K_Ds in Figure 2). Based on this behaviour, we used doubly phospho-mimetic CENP-T (T72D and S88D) for further assays in this paper. We have described this point in the text of the revised version.

7- *Page 10 and Figure 5: The authors report that the binding of CENP-T and Mis12 to Ndc80 is mutually exclusive. This is surprising in light of data contained in one of the authors' previous papers (Gascoigne et al., Cell 2011, Figure 4) that CENP-T binds Mis12. Here such interaction seems to disappear and is replaced by a competitive interaction with Ndc80. The authors should clarify this apparent discrepancy and clearly refer to it in the text.*

As this reviewer mentioned, we have previously shown that the Mis12 complex can interact with CENP-T based on pull-down assays (Gascoigne et al., Cell, 2011). In that case, we detected small amounts of the Mis12 complex by Western blot analysis. In the experiments presented in this paper, we did not detect co-migration of the Mis12 complex with CENP-T by gel filtration (Figure 5E and S2C). Based on this, the Mis12 complex does not strongly associate with CENP-T. We have described this point in the revised version.

8- *Page 11: "...to provide a distinct pathway for outer kinetochore assembly (Hori et al., 2008; Gascoigne et al., 2011)." Neither citation is correct. The demonstration was first provided by the Przewloka et al. 2011 paper in Current Biology (incidentally, these authors showed that the CENP-C-Mis12 interaction is the crucial interaction in Drosophila, where CENP-T is probably absent). Second, there is no evidence that the pathways are distinct.*

Based on this suggestion, we carefully edit this sentence in the revised version.

9- *Page 12: "In total, we conclude that the Mis12 complex binds to the Spc24/25 complex using distinct interaction residues from CENP-T, and the binding of the CENP-T N-terminus and the Mis12 complex to the Spc24/25 is mutually exclusive likely due to steric occlusion of this interaction surface". This formulation is slightly confusing, but once again, if the interaction is competitive, is it because the binding sites of Spc24/25 for Mis12 and CENP-T partly overlap? Or not?*

As we described above, we have now measured the binding affinity of the mutant Spc24/25 complex with the Mis12 complex by ITC. We conclude that it is possible that the binding sites of Spc24/25 for Mis12 and CENP-T partially overlap. Based on these new data, we have modified this sentence in the revised version.

10- Page 12-13 and Figure 6: The experiment is inconclusive. There is no quantitative understanding for how mutations on the CENP-T binding interface of Spc24/25 affect Mis12 binding. Once again, if the binding of CENP-T and Mis12 to Spc24/25 is competitive, most likely the mutations on Ndc80 affect Mis12 binding too, so that the observed reduction of Ndc80 at kinetochores might not simply reflect loss of CENP-T binding. To be rigorous, the authors should measure the binding affinity of Mis12 to Bonsai and mutant Bonsai and show that they are identical. Only then they will be able to support their claim. Co-elution in gel filtration experiments is not sufficient, because there might be sufficient residual binding affinity of Mis12 to the Ndc80 complex, and yet this might not be sufficient in cells.

We have now quantified the protein levels in the revised Figure 6 and Figure S6. Although we conclude that it is possible that the binding sites of Spc24/25 for Mis12 and CENP-T partially overlap, the Mis12 complex must also use other sites of the Spc24/25 complex for the interaction, because the mutant Spc24/25 complex (I149A_L154A for Spc25) still binds to the Mis12 complex (Figure 5D and S5C).

11- Discussion: There are other instances of main chain stabilization by phosphorylation (e.g. Nature 430, 223-226 (8 July 2004) Recognition of RNA polymerase II carboxy-terminal domain by 3'-RNA-processing factors, Anton Meinhart & Patrick Cramer)

We appreciate this comment and have cited this reference in the revised version.

Reviewer #3

1- What level of NDC80 is recruited to kinetochores in the absence of a functional CENP-T recruitment site? The answer to this question is not clear: throughout the paper, there are contradicting images, quantitative values, and estimations. The level of NDC80 at kinetochores under each of the experimental conditions should be quantitated. For example, in Figure 1C, the authors report that only 3.4% of NDC80 is left at kinetochores after expressing a CENP-T del69-90 fragment. However, later in the paper (Figure 6, Supp Figure 5), the authors claim that preventing CENP-T-based recruitment only reduces NDC80 at kinetochores by an estimated 50%. Where does this estimation come from? Why are these values so different?

For the previous version, we measured Ndc80 intensity at kinetochores in CENP-T Δ69-90 cells at 120 hours following tet addition. At this stage, many cells had died, complicating this analysis. For the revised version, we have now measured Ndc80 intensity in CENP-T Δ69-90 cells 72 hours after tet addition. In this case, the intensities were reduced to 41% of control levels (new Figure 1C). In addition, we measured the intensity of Ndc80 in CENP-T- and DsnI-degron cells (Figure S6). In this case, Ndc80 intensity was reduced to 43% and 32%, respectively after degradation of the target proteins. As Ndc80 was still visible at 30-40% levels in both CENP-T- and DsnI-deficient cells, we emphasise that there are two parallel pathways for recruiting the Ndc80 complex to kinetochores.

2- Related to this point, if there are two parallel pathways for NDC80 recruitment, why don't we see NDC80 at kinetochores in Figure 1C (presumably the CENPT pathway is inactive, but shouldn't Mis12C compensate?).

As we described above, we now detect Ndc80 at 41% of control levels in CENP-T Δ69-90 cells (revised Figure 1C). This data supports a model of two parallel pathways for Ndc80 recruitment.

3- Again, in Figure 6A, the authors present data suggesting that cells expressing the Spc mutant (that cannot bind CENP-T) have some residual levels of NDC80 at kinetochores. How much is there? In the images it looks like very little, but in Figure 6C, this level is clearly enough to support survival of the cells..

We measured the intensity of Spc25 (I156R) at kinetochores and find that the level was reduced to 60% of wild type Spc25 (new Figure 6A). We are confident that Spc25 levels at kinetochores are reduced, but we emphasize that this level of Spc25 is sufficient to allow cell proliferation, as cells in which wild-type Spc25 was replaced with Spc25 (I156R) mutant are still viable. We have described this point in the revised version.

4- *Related to the above point: in the graph in Figure 6, the difference between survival in tet on and tet off cells looks very minor. Is this significant?*

Although cells in which wild-type Spc25 is replaced by the Spc25 (I156R) mutant are viable (+tet), their growth rate is reduced. The doubling time of these cells is 14.2 h, whereas control cells double every 13.1 h. As the growth curve in the previous version was a bit unclear, we have now provided this data for the doubling time in the Figure legend.

5- *Why is a CENP-T deletion lethal (Figure 1E), but cells expressing the Spc mutant (that cannot bind CENP-T) survive and proliferate (Figure 6C)? Do the authors suppose that CENP-T has some additional function not related to recruitment of NDC80 to kinetochores?*

We demonstrate that Spc25-deficient DT40 cells are lethal (Figure S6). However, in the Spc25 (I156R) mutant, 60% of Spc25 still localizes to kinetochores and the mutant cells are still viable. If the level of Spc25 were further reduced, the cells would die. In addition, we previously demonstrated that CENP-T has a DNA binding activity in C-terminal domain (Nishino et al., Cell, 2012) and it is highly likely that CENP-T has additional functions that are unrelated to Ndc80 recruitment. We have tried to emphasize both of these points in the text.

6- *It is unclear how the authors envision the recruitment of NDC80 by two parallel pathways. Based on the proposed phospho -regulation of CENP-T/NDC80 binding, in early mitosis is CENP-T the major receptor for NDC80? As mitosis progresses and CENP-T is dephosphorylated, does NDC80 switch to Mis12 binding? Have the authors tested if a phospho-mimetic mutant of CENP-T affects mitotic progression (to prevent "release" of NDC80 from CENPT)? Some discussion of how these two parallel pathways may function in mitosis would be helpful.*

We appreciate this comment. We have described our ideas for these two pathways in the Discussion of the revised version. We have replaced CENP-T with the phospho-mimetic mutant in both human and DT40 cells. However, we did not observe strong defects in mitotic progression. In contrast, we have previously published that phospho-inhibitory CENP-T mutants severely compromise kinetochore assembly (Gascoigne et al., Cell, 2011). As CENP-T is phosphorylated during mitosis and Ndc80 binds to the Mis12 complex during mitosis, it is likely that the two parallel pathways are partially redundant in vertebrate cells. However, it is also possible that these two populations of Ndc80 play distinct functions. *C. elegans* and *D. melanogaster* lack CENP-T and only have the Mis12 pathway. In this case, it may be sufficient to have single pathway to recruit Ndc80. However, as many species have two pathways, the presence of these separate pathways may strengthen the connection between the inner and outer kinetochores. In addition, although we propose that there are two parallel pathways, there may be some connection between these pathways because depletion of either CENP-T or Mis12 shows defects for functional kinetochore formation and CENP-T depletion reduces the localization of Mis12 and KNL1.

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Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by one of the original referees (see comments below), and I am happy to inform you that there are no further objections towards publication in The EMBO Journal.

Before we will able to send you a formal letter of acceptance, I need to ask you for a revised version of Figure 3 taking into account the remaining presentational concern of referee 2 (see below).

After that, we should then be able to swiftly proceed with formal acceptance and production of the manuscript!

Referee #2

(Remarks to the Author)
I wish to thank the authors for a through review of their manuscript. The manuscript is now ready for publication. Contrarily to what is written in their rebuttal, the authors have not changed the colors in Figure 3. In panel A, they represent Spc24 and Spc25 in purple and light green, respectively, and CENP-T in red. In panel B they use two different tones of green/cyan for Spc24 and Spc25 (and as far as Spc25 is concerned, they use a dark tone of green different from the lighter one used in panel A) and now CENP-T is shown in purple. The two tones of green/cyan for Spc24 and Spc25 are essentially indistinguishable in the printout. Why not try to harmonize this figure? Using the same colors in A and B and sufficiently different colors for Spc24 and Spc25 will help readers.

