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Mad1 contribution to spindle assembly checkpoint signalling goes beyond presenting Mad2 at kinetochores

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

Editor: Nonia Pariente

1st Editorial Decision 22 November 2013

First of all, I am sorry that the peer-review process has been longer than usual, as one of the referee reports was rather tardy. We now have received a full set of reports, which you will find below, and I have had time to go through them in detail. As you will see, although all the referees find the topic of interest and the work elegant and overall technically sound, they consider the reported advance relatively limited and all point out various issues that would need to be addressed before we can consider a revised version of the study.

As the reports are very detailed, I will not belabor them here. Notably, it would be important to clarify whether Bub1 binding by Mad1 is necessary for checkpoint activity, which will help the study break new ground and conceptually move forward from previously published data. In addition, referees 2 and 3 raise also a number of technical issues, all of which would need to be addressed during revision.

As you may know, it is EMBO reports policy to undergo one round of revision only and thus, acceptance of your study will depend on the outcome of the next, final round of peer-review.

Revised manuscripts must be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Revised manuscript length must be a maximum of 28,500 characters (including spaces). When submitting your revised manuscript, please also include editable TIFF or EPS-formatted figure files, a separate PDF file of any Supplementary information (in its final format) and a letter detailing your responses to the referees.

We also welcome the submission of cover suggestions or motifs that might be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised form of your manuscript when it is ready. In the meantime, do not hesitate to get in touch with me if I can be of any assistance.

REFEREE REPORTS:

Referee #1:

In this concise and generally convincing manuscript Hauf and colleagues demonstrate that Mad1 has an additional role in the spindle checkpoint beyond its role in recruiting Mad2 to the kinetochore. The observation is important. It is also somewhat foregone and not completely novel. It is important because it strongly suggest that the checkpoint proteins need to interact physically at the kinetochore rather than simply becoming recruited there. In turn, this suggests that there is a larger macromolecular complex operating at the kinetochore, in which Mad1, Mad2, and Bub1 come together to create the MCC at high rates. Why the observation might be foregone and not completely novel is explained below.

The work is of the highest technical quality and the results are clearly presented. The literature is accurately cited. If the authors made at least a quick attempt to test the main hidden hypothesis of the paper, that Mad1 needs to bind Bub1 for the checkpoint to work, the expected impact of the paper would be greatly increased.

Specific points

Abstract
The final claim that the Mad1 CTD has a previously unrecognized role in the checkpoint is not giving Hardwick's work proper credit. It is true that Brady and Hardwick carried out their studies without any detailed structural information on the Mad1-Mad2 complex, but they demonstrated that mutations in the CTD affect the interaction of Mad1 with Bub1 and have an effect on the checkpoint. So I would argue that the novelty claim is unjustified and that although much better articulated and significantly expanded, the work is confirmatory in its outline.

End of introduction
The authors claim that "Hence, the Mad1 C-terminus is not only required for bringing Mad2 to kinetochores, but has an additional, previously unrecognized role promoting checkpoint activity." I would like to argue that this is hardly surprising. When a protein is recruited to a site in the cell by a given receptor, most likely it interacts with that receptor to perform its function. Bringing the protein to the same site in the cell in the absence of the receptor, or after inserting mutations that prevent it from interacting with the receptor, most likely prevents functional complementation. The reason why I mention this here is that it seems to me that the authors' data are consistent with one hypothesis, i.e. that Bub1 recruits Mad1, which is known, and that the interaction is important for the checkpoint, a hypothesis that needs substantiation. It is unclear to me why the authors don't discuss this simple hypothesis in the final paragraph of the manuscript. The key experiment to test this would be to create an artificial dimerizer (either chemical or by using the "zippers" described in Andrew Murray's recent paper on Mad2-Cdc20 dimerization in Current Biology) linking the Mad1 mutant to Bub1, so to force the two to interact. If the checkpoint response were restored, it will be proved that the Mad1 C-terminus needs to interact with Bub1 for the checkpoint to function. Is this technically unfeasible? Of course there are many reasons why this experiment might fail, but if it worked it would prove what seems to be the main hidden message of the paper.

Referee #2:

This study reports an interesting series of mutations in the fission yeast Mad1 checkpoint protein. Mutations within the C-terminus perturb the checkpoint, even though they don't impair Mad2 binding and can be artificially tethered to kinetochores (via Mis12 fusion). It is argued that Mad1 must have another function, in addition to Mad2 binding/KT recruitment.

Neither this study, nor the accompanying Nilsson study, have identified the new Mad1 function that is dependent on the Mad1 C-terminus. Both studies are interesting, and of high technical proficiency, but neither significantly advance our mechanistic understanding of the SAC. They do however, clearly point out that Mad1 has roles to play in addition to Mad2 recruitment to KTs. This is not an entirely new finding: several reports have shown that Mad1 has a more severe loss of function phenotype than loss of Mad2 (yeast genetics) and that Mad1 has "additional mitotic functions" (yeast and fly genetics). This is however a nice, thorough study and will serve to remind the field of the importance of Mad1.

Specific points:
Abstract: Mad1 has a previously unrecognised "active" role. It is not clear what is meant here by "active".

Bub1-cm1 mutant (p5): odd wording is used here about Mad2 localisation "and therefore presumably Mad1" (why not look directly at Mad1?). How was S381A, T383A, T386A chosen as the specific mutation to make in Bub1? In the paper referenced, studying vertebrate Bub1, a deletion was made of this conserved domain. Is it significant serines and threonines are being mutated here? Have other, nearby mutations also been tested?

Bottom p6: Mad2 to Mad1 ratio is reduced in R133A - but not by 50%? Why not? Is the Mad2 recruited by the tethered Mad1-RLK dynamic?

Page 7: Mad1 is described here as an "active" player and having an "enigmatic role". Couldn't this role simply be Bub1 binding (as previously described for the RLK region in budding yeast)? Could the authors test this directly, using 2-hybrid assays for example?

Intra and inter-molecular interactions are mentioned but are not clear - what is the binding partner? Mad1 itself (in the dimer) or another protein?

Figures:
Figure legends throughout are far too brief.

Fig. 1B: more needs to be said of these Mad1 images. There appears to be signal on/near the spindle in several panels (all apart from the AAA?). What is this - SPBs, spindles or some KT signal.
Fig. 1C: would be good to see a control here to know when in the tiome sourse we should expect to see a KT signal if there were one. Compare Mad3-GFP through the same time course.
Fig. 1D: these images are not correct - they appear to be Ark1!?
Fig 1I: it would be better to show Mad1-cherry (rather than Mad2 here).

Fig 2B: there is a sub-set of movies where the Mis12-Mad1-GFP strain exits mitosis fast - the authors should comment on this perturbation of the checkpoint. How happy are they with this artificial fusion protein?

Fig 2D images: again there are multiple signals in the nucleus for the wt Mad1-GFP. Are these KTs and spindle poles? Is this cell later in mitosis? Do the authors believe Mad1 has a function at spindle poles? Could this be the enigmatic function?

Fig 3G: there are large error bars here. N=7 and should be increased (to 20?)

Referee #3:

The spindle checkpoint ensures the fidelity of chromosome segregation by delaying anaphase onset until all chromosomes are properly attached to spindle microtubules. Mad1 and Mad2 are both critical downstream components of this checkpoint. Mad2 is an unusual two-state protein: the active C-Mad2 and the inactive O-Mad2. It is believed that a Mad1-C-Mad2 core complex needs to be recruited to unattached kinetochores, where it "catalyzes" the efficient conversion of cytosolic O-Mad2 to active C-Mad2.

In this manuscript, the authors show that the conserved RLK motif in the C-terminal domain (CTD) of the S. pombe Mad1 is critical for the kinetochore localization of Mad1, but is not required for the Mad1-Mad2 interaction. Mutations in the Mad1 RLK motif abolish the spindle checkpoint. Consistently, mutations of the putative RLK motif-binding region on Bub1 also cause Mad2 delocalization from kinetochores and a defective spindle checkpoint. Furthermore, a Mis12-Mad1-RLK/AAA fusion protein (which localizes to kinetochores) restores Mad2 kinetochore localization, but cannot restore the spindle checkpoint. These data indicate that the RLK motif has a checkpoint role in addition to targeting Mad1-Mad2 to kinetochores. Finally, mutations of the extremely C-terminal helix of Mad1 also abolish checkpoint signaling, without disrupting the Mad1-Mad2 interaction and their kinetochore localization. Taken together, their results suggest that Mad1 CTD has a more direct role in the spindle checkpoint, aside from recruiting the tightly bound C-Mad2.

Overall, the manuscript contains several interesting findings. Most of the results are solid, with the few exceptions noted below. These specific points need to be addressed. In addition, the new function of Mad1 CTD has not been mechanistically characterized. The model in the end is highly speculative. This speculation needs to be toned down. If these issues can be addressed, publication in EMBO Reports is recommended.

Minor points:

(1) In Fig. 1, the Bub1 cm1 mutant had normal kinetochore localization, but severely reduced Mad2 localization on kinetochores. Does this mutant also reduce Mad1 kinetochore localization, as would be expected?

(2) The authors state that Mad1Δhelix keeps Mad1-Mad2 binding intact. In Fig. 3F, Mad2 binding to Mad1Δhelix appeared to be weakened. The conclusions drawn from Δhelix mutant need to be revised.

(3) It is stated that the Mad2 R133A mutation causes a reduction of Mad2/Mad1 ratio and Mad2 kinetochore signal (Fig3. G and H). Statistic analysis of both sets of data is required to support their statement. In addition, this statement would be further bolstered if they can show a reduced Mad2 signal in Mad1 IP.

(4) There is an inconsistency in the phenotype of the Mad1 fragment 458-676. In Fig. S2 C and E, fragment 458-676 shows reasonable kinetochore localization, similar to WT and 306-676; however, in panel D, the kinetochore signal of the same fragment is somewhat defective. Why is this?

Correspondence - authors 23 November 2013

Thank you very much for your decision letter. We were happy to read that you and the reviewers found the study interesting and well performed. We are confident that we can address all the remaining concerns. (Some of the required experiments have already been performed in the meanwhile.)

However, there is one issue that I would like to discuss with you:
We are in a little bit a difficult situation because of the move of my lab to the United States in mid-

December. We are able to work full time until then, but because all the equipment is being shipped (which takes 4-6 weeks), we will then not be able to perform any experiments until February.
We think we can perform all crucial remaining experiments for this manuscript by mid-December, but we will not be able to start new series of experiments - as we would probably have done with ample time at hand. In particular, the artificial linkage of Mad1 and Bub1 requested by reviewer #1 will not be doable by then.

However, we do have reason to believe that this experiment may not be informative:
Reviewer #1 points out that one of the most likely defects in the Mad1 C-terminal mutants is a failure to interact with Bub1. We would have thought so, too. However - unlike in budding yeast, where the interaction between Mad1 and Bub1 is obvious and can be seen by IP - we have been unable to detect an interaction between the two proteins. This is based on sensitive mass spec experiments that allow us to see all other protein-protein interactions that are important in checkpoint signaling, and is true for both Mad1 and Bub1 IPs. Jakob Nilsson's laboratory has the same observation for human cells.
Since we do not observe interaction for the wild type proteins, we cannot check wether interaction is perturbed in the mutants.

Furthermore, in the specific Mad1 mutants that we created (EDD/QNN and delta-helix), which are checkpoint-defective, Mad1 localization to the kinetochore is preserved, and Bub1 localization is very likely preserved as well (because Mad1 requires Bub1 for localization - but we will double-check by visualizing Bub1). This means that both Mad1 and Bub1 localize at their natural place, yet the checkpoint is inactive. The remaining possibility is that - despite the proper localization - the mutations in Mad1 abolish a weak and transient interaction with Bub1 specifically at kinetochores, thereby causing the defect. Since the proteins are at their native place and should in principle have the opportunity to interact, we consider it unlikely that an artificial connection between the two proteins, which would perturb their native, relative position at kinetochores, would result in any effect.

Nevertheless, we do have evidence that actually supports the reviewers' view (and which is currently only partly shown in the manuscript):
Since Mad1 requires Bub1 for enrichment at the kinetochore, and this enrichment is abolished in the Mad1-RLK/AAA mutant, we bypassed this recruitment function of Bub1 by artificially binding Mad1 to the kinetochore.
Surprisingly, checkpoint function is not restored when tethering Mad1-RLK/AAA in cells that have Bub1 (Figure 2B) nor when tethering wild type Mad1 to cells that lack Bub1 or that express Bub1-cm1, which itself localizes to kinetochores. (The latter data is currently not shown).
Therefore, in both wt-Bub1+tethered Mad1-RLK/AAA or in Bub1-cm1+tethered wt-Mad1, both Mad1 and Bub1 are at kinetochores, but some step in signalling does not work (similar to wt-Bub1+Mad1-EDD/QNN or delta-helix). This could therefore indeed indicate that Bub1 and Mad1 are connected to allow signalling. Yet, since both proteins are at kinetochores, simply artificially tethering them is unlikely to rescue the defect.

We would have loved to address this interplay between Bub1 and Mad1 mechanistically, but this will be extremely difficult. This is why we decided to submit this manuscript as is - so that more people can see the data and come up with ways of tackling this issue.

I want to point out that our observations go well beyond the observations by Brady and Hardwick that reviewer #1 refers to. This should by no means depreciate this paper, which contains very important information and was a huge step forward at that time. The most straightforward interpretation from the Brady/Hardwick data is that the Mad1-RLK motif is required for interaction with Bub1 (shown by them) and that this interaction is required for bringing Mad1 to the kinetochore (our ms. and Kim et al. 2012), and therefore for signalling. I believe that Sue Biggins' lab has a manuscript (probably by now submitted or accepted) that shows precisely that.
Yet, as we show through the tethering experiments and through the more specific Mad1 mutants (EDD/QNN and delta-helix), this is only part of the story. Even when both Mad1 and Bub1 are at kinetochores, signalling fails if the Mad1 C-terminus or the conserved motif part of Bub1 is not functional. This can only be gleaned from our experiments, but not from the Brady/Hardwick (or the more recent Biggins lab) data.

Under the given circumstances, I would therefore like to propose that we re-submit the manuscript towards the end of December with all crucial technical points addressed, but without further exploration whether an artificial linkage between Bub1 and Mad1 can bypass the checkpoint defects of the Mad1 C-terminal separation of function mutants (EDD/QNN and delta-helix).

Please let me know whether you find this in principle acceptable.

Again, thank you for your consideration of our manuscript and your careful assessment.
I am looking forward to hearing from you.

Correspondence - editor 25 November 2013

Many thanks for your detailed letter. I appreciate your time constraints and think that the plan you outline would sufficiently address referee 1's point.

I look forward to receiving a revised version of your study at the end of the year then.

1st Revision - authors' response 20 December 2013

Referee #1:

In this concise and generally convincing manuscript Hauf and colleagues demonstrate that Mad1 has an additional role in the spindle checkpoint beyond its role in recruiting Mad2 to the kinetochore. The observation is important. It is also somewhat foregone and not completely novel. It is important because it strongly suggest that the checkpoint proteins need to interact physically at the kinetochore rather than simply becoming recruited there. In turn, this suggests that there is a larger macromolecular complex operating at the kinetochore, in which Mad1, Mad2, and Bub1 come together to create the MCC at high rates. Why the observation might be foregone and not completely novel is explained below.

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Specific points

Abstract
The final claim that the Mad1 CTD has a previously unrecognized role in the checkpoint is not giving Hardwick's work proper credit. It is true that Brady and Hardwick carried out their studies without any detailed structural information on the Mad1-Mad2 complex, but they demonstrated that mutations in the CTD affect the interaction of Mad1 with Bub1 and have an effect on the checkpoint. So I would argue that the novelty claim is unjustified and that although much better articulated and significantly expanded, the work is confirmatory in its outline.

> We have now highlighted better what was known through the undoubtedly important Brady and Hardwick study. However, this study is still consistent with the hypothesis that the Bub1-Mad1 interaction is required for bringing Mad1 to kinetochores and therefore for checkpoint signalling. We think the novelty of our study is in showing that specific mutations within Mad1 abrogate checkpoint signalling even when Bub1, Mad1 and Mad2 all localize (naturally) to kinetochores. This goes well beyond what was known previously. It would be interesting to know whether these specific mutants have some defect in Bub1 interaction. However, since we are unable to detect an interaction between wild type Bub1 and Mad1 in fission yeast (see below), this has not been possible to test.

End of introduction
The authors claim that "Hence, the Mad1 C-terminus is not only required for bringing Mad2 to kinetochores, but has an additional, previously unrecognized role promoting checkpoint activity." I would like to argue that this is hardly surprising. When a protein is recruited to a site in the cell by a given receptor, most likely it interacts with that receptor to perform its function. Bringing the protein to the same site in the cell in the absence of the receptor, or after inserting mutations that prevent it from interacting with the receptor, most likely prevents functional complementation.

> We agree with the reviewer that it is conceivable that failure to properly interact with a kinetochore receptor also leads to a defect in function. However, in the context of the spindle assembly checkpoint, specific types of interactions at the kinetochore are at least not always required. For example, an Mps1/Mph1 mutant that has lost its capability to naturally interact with the kinetochore provides checkpoint function when artificially tethered (i.e. in the absence of interaction with its endogenous receptor) (Nijenhuis, JCB 2013; Heinrich, JCS 2012). Similarly, destroying the Bub1-Bub3 receptor by specific mutations in the kinetochore protein Spc7 is overcome by artificial recruitment of Bub1 (Yamagishi, NCB 2012). Hence, the situation that is described by the reviewer may be the exception rather than the rule.
Most importantly, we demonstrate that specific mutations within Mad1 (EDD/QNN and Δhelix) preserve kinetochore localization of Bub1, Mad1 and Mad2 (so the endogenous interactions that are required for recruitment should be preserved). Yet, these mutants fail to support checkpoint signalling.

The reason why I mention this here is that it seems to me that the authors' data are consistent with one hypothesis, i.e. that Bub1 recruits Mad1, which is known, and that the interaction is important for the checkpoint, a hypothesis that needs substantiation. It is unclear to me why the authors don't discuss this simple hypothesis in the final paragraph of the manuscript. The key experiment to test this would be to create an artificial dimerizer (either chemical or by using the "zippers" described in Andrew Murray's recent paper on Mad2-Cdc20 dimerization in Current Biology) linking the Mad1 mutant to Bub1, so to force the two to interact. If the checkpoint response were restored, it will be proved that the Mad1 C-terminus needs to interact with Bub1 for the checkpoint to function. Is this technically unfeasible? Of course there are many reasons why this experiment might fail, but if it worked it would prove what seems to be the main hidden message of the paper.

> We agree with the reviewer that a prime hypothesis is an interaction between Mad1 and Bub1 that may not only be required for kinetochore localization, but also for making Mad1 and Bub1 proficient for checkpoint signalling. However, we failed to point out in the previous version of the manuscript that we are unable to see any interaction between Bub1 and Mad1 in cells with an active checkpoint by co-immunoprecipitation. This is using sensitive mass spectrometry that shows us known interactors of Bub1 and Mad1 as well as interactions of Bub1 with kinetochore proteins. We now mention this in the text and show the (negative) data in Figure S3. This is in contrast to the results by Brady and Hardwick in budding yeast (which have been confirmed by Sue Biggins' lab). We cannot explain this discrepancy at the moment. However, also in human cells, it seems to be very difficult/impossible to detect this interaction so far (Kim, PNAS 2012; Nilsson and Kops groups, personal communication).

We agree that it is nevertheless possible that a weak Mad1-Bub1 interaction at kinetochores (that we are unable to detect) is required and may be perturbed in the Mad1 mutants. We want to point out however that in the specific C-terminal Mad1 mutants that we created (Mad1-EDD/QNN and Mad1-Δhelix) both Bub1 (now newly shown in Figure S4G) and Mad1 are present at kinetochores – presumably at their natural position. (They are not artificially targeted.) Yet, checkpoint function is strongly impaired. Because the proteins are – very likely – at their natural place in this situation, we do not think that fusing the two proteins in an artificial way would be likely to rescue this defect.

We will nevertheless attempt this fusion in the future. However, to be very honest, we cannot do the experiment at present in a reasonable time frame because my laboratory is in the process of moving from Europe to the US. All our equipment is currently being shipped, and we will only be able to do experiments again in February. Since we did not have the required strains ready it was impossible for us to complete this experiment before moving.

Referee #2:

This study reports an interesting series of mutations in the fission yeast Mad1 checkpoint protein. Mutations within the C-terminus perturb the checkpoint, even though they don't impair Mad2 binding and can be artificially tethered to kinetochores (via Mis12 fusion). It is argued that Mad1 must have another function, in addition to Mad2 binding/KT recruitment.

Neither this study, nor the accompanying Nilsson study, have identified the new Mad1 function that is dependent on the Mad1 C-terminus. Both studies are interesting, and of high technical proficiency, but neither significantly advance our mechanistic understanding of the SAC. They do however, clearly point out that Mad1 has roles to play in addition to Mad2 recruitment to KTs. This is not an entirely new finding: several reports have shown that Mad1 has a more severe loss of function phenotype than loss of Mad2 (yeast genetics) and that Mad1 has "additional mitotic functions" (yeast and fly genetics). This is however a nice, thorough study and will serve to remind the field of the importance of Mad1.

Specific points:
Abstract: Mad1 has a previously unrecognised "active" role. It is not clear what is meant here by "active".

> We used the word 'active' as a contrast to the 'passive' function of merely being a scaffold for Mad2. However, since this apparently was confusing we removed it from title and abstract, but still use the passive/active distinction in the discussion.

Bub1-cm1 mutant (p5): odd wording is used here about Mad2 localisation "and therefore presumably Mad1" (why not look directly at Mad1?).

> We previously did not have a strain available to check Mad1 localization – and therefore used the indirect assay via Mad2 (hence the odd wording). We now demonstrate that Mad1 localization is strongly impaired by the *bub1-cm1* mutation (new Fig 1J,K) and that – in addition to Bub1 itself – the localization of Bub3 and Mad3 is preserved (supplementary Fig S1I).

How was S381A, T383A, T386A chosen as the specific mutation to make in Bub1? In the paper referenced, studying vertebrate Bub1, a deletion was made of this conserved domain. Is it significant serines and threonines are being mutated here? Have other, nearby mutations also been tested?

> The sites were chosen based on conservation across species. A protein sequence alignment is now provided in supplementary Fig S1E. For unknown technical reasons (already in the PCR step required for creating the mutant), we have been unable to delete the entire conserved motif. We have tested additional point mutations: K387E did not cause any checkpoint defect and I384Q A389Q I393Q F397Q caused a checkpoint defect but also impaired the stability of the protein. We therefore chose to only show the STT/AAA triple mutant.

Bottom p6: Mad2 to Mad1 ratio is reduced in R133A - but not by 50%? Why not? Is the Mad2 recruited by the tethered Mad1-RLK dynamic?

> A reduction to 50 % would only be expected if each and every Mad1-bound Mad2 was occupied by a second Mad2. It is not known whether this is the case.
Furthermore, it is not possible to read the precise ratio from the data, since this is not an absolute quantification (i.e. we do not know how the mCherry and GFP intensities relate to molecule number). The autofluorescence contribution, which we do not subtract in this experiment, distorts the ratio.
We attempted to address whether the dynamics of Mad2 recruitment remain similar, but the required FRAP experiments are extremely challenging in yeast cells. The low endogenous intensities of the checkpoint proteins are very difficult to detect in confocal microscopy, it is difficult to avoid unintended bleaching and therefore difficult to track the moving kinetochores after the intended bleaching. We therefore unfortunately do not have conclusive data.
To further corroborate that Mad2 dimerization is intact in the Mad1-EDD/QNN and -Δhelix mutant we now combined these mutations with the Mad2-R133A mutation and show that the Mad2/Mad1

ratio at kinetochores is lower with Mad2-R133A than with wild type Mad2 (Fig 3G). This is a further indication that dimerization of wild type Mad2 is not impaired in these Mad1 mutants.

Page 7: Mad1 is described here as an "active" player and having an "enigmatic role". Couldn't this role simply be Bub1 binding (as previously described for the RLK region in budding yeast)? Could the authors test this directly, using 2-hybrid assays for example?

> Yes, we agree that this was one of the prime hypotheses. We failed to point out in the previous version of the manuscript that we are unable to detect an interaction between Mad1 and Bub1 in cells with an active checkpoint (now shown in supplementary Fig S3). The situation seems to be similar in human cells (Kim, PNAS 2012; Nilsson group and Kops group, personal communication). Because *bub1-cm1* and *mad1-RLK/AAA* mutants have a similar phenotype, we are puzzled by this result, and we still consider it possible that there is a weak or kinetochore-specific interaction that we are unable to detect. We now discuss this better in the text.

Intra and inter-molecular interactions are mentioned but are not clear - what is the binding partner? Mad1 itself (in the dimer) or another protein?

> We have now shortened the respective paragraph in the main text and explain (hopefully) better what we mean in the legend to Figure 4.

Figures:
Figure legends throughout are far too brief.

> We have now made an effort to provide more helpful legends and in particular to explain better what is seen in the pictures (see below).

Fig. 1B: more needs to be said of these Mad1 images. There appears to be signal on/near the spindle in several panels (all apart from the AAA?). What is this - SPBs, spindles or some KT signal.

> The images only show the nucleus of *S. pombe* cells. These cells are in early mitosis, which can be judged from the localization of Plo1-mCherry to spindle pole bodies and from the short spindle. At this early time in mitosis, chromosomes are still in the process of attaching to the spindle, so that many kinetochores are decorated with checkpoint proteins. In this particular assay it is impossible to discern single chromosomes. The evidence that the staining is on kinetochores comes from previous ChIP experiments and from experiments where the spindle is destroyed so that single chromosomes/kinetochores can be seen.
When microtubule formation is avoided (by the tubulin mutation *nda3-KM311* at the restrictive temperature of 16 ºC), the spindle pole bodies do not separate (e.g. Fig 1E). As long as cells are in early mitosis, kinetochores cluster close to the spindle pole bodies, so that in general only one dot for spindle pole bodies and one dot for kinetochores is seen. If cells are delayed in mitosis, kinetochores uncluster from spindle pole bodies over time, which is the situation seen in supplementary Fig S2D.
We now show schematics in Fig 1B and supplementary Fig S2D. We hope this helps to understand the data.

Fig. 1C: would be good to see a control here to know when in the time course we should expect to see a KT signal if there were one. Compare Mad3-GFP through the same time course.

> As a control, we show the signal for wild type Mad1-GFP. This is increasing as cells enter mitosis (black circles in upper and lower part). In contrast Mad1-GFP signals in the mutants are not increasing, showing the failure to accumulate at kinetochores. We knew that the cells were entering mitosis because we monitored appearance of Plo1-mCherry at SPBs. Since we only have two markers available for live cell imaging (GFP and mCherry), we cannot visualize Plo1, Mad1 and yet another checkpoint protein at the same time. However, Fig 1E and S1B show that other checkpoint proteins can be seen at kinetochores in the *mad1-RLK/AAA* mutant.

Fig. 1D: these images are not correct - they appear to be Ark1!?

> Thank you for having spotted this! Yes, we made a copy/paste mistake and the color-merged picture shown for *mad1-RLK/AAA* Mad2-GFP was incorrect. It has now been replaced.

Fig 1I: it would be better to show Mad1-cherry (rather than Mad2 here).

> Yes. We did not have the appropriate strain available at that time. We now show that Mad1 (new Fig 1J,K) as well as Mad2 localization (Fig. S1I) are perturbed in the *bub1-cm1* mutant, whereas localization of Bub1 (Fig 1K), Bub3 and Mad3 (new Fig S1I) is preserved.

Fig 2B: there is a sub-set of movies where the Mis12-Mad1-GFP strain exits mitosis fast - the authors should comment on this perturbation of the checkpoint. How happy are they with this artificial fusion protein?

> The reviewer rightly points out that the Mis12-Mad1-GFP construct is not fully functional. The degree to which the checkpoint arrest works is variable between experiments (see new Figure 2B,D), partly because expression from the inducible *P(nmt81)* promoter is not always identical. We have also found that under certain conditions the C-terminal tag on Mad1 seems to slightly perturb function. Nevertheless, there is a drastic difference between the tethered wild type and tethered mutant protein, and we therefore think that our conclusions are justified. We have revised the text to clarify that the wild type construct is not fully functional.

Fig 2D images: again there are multiple signals in the nucleus for the wt Mad1-GFP. Are these KTs and spindle poles? Is this cell later in mitosis? Do the authors believe Mad1 has a function at spindle poles? Could this be the enigmatic function?

> As we now try to show in the schematics (Fig 1B), *S. pombe* chromosomes are so close together in early mitosis that often only one signal for all kinetochores can be seen. However, sometimes up to three signals (for the three chromosomes) can be seen, and in this particular wild type picture, two were visible. In mutants that do not delay in mitosis (because the checkpoint is defective), it is rarer to see several signals. In order to compare wild type and mutants, we therefore typically focus on early mitotic cells with one signal for all chromosomes. In order to avoid confusion, we have now picked a different example for a wild type cell.
It is true that Mad1 and Mad2 show very weak spindle pole body localization (much weaker than the kinetochore signal). This is particularly prominent in anaphase (when the checkpoint is not actively signalling anymore). We did not observe any obvious difference in spindle pole body localization in the Mad1 mutants. This does not exclude a function at SPBs that is perturbed in the checkpoint-inactive Mad1 mutants, but we also do not have any particular indication that this might be the case.

Fig 3G: there are large error bars here. N=7 and should be increased (to 20?)

> It is difficult to reach high numbers in these experiments. A prominent problem is that we are restricted to analysing very early mitosis (because the checkpoint-deficient strains exit mitosis quickly, and we only want to consider wild type cells at the same stage). For better comparability, we also restrict ourselves to early mitotic cells that have not (yet) unclustered the chromosomes from the spindle pole bodies. In addition, we need cells to stay in focus throughout the experiment, which further limits the number of cells. (This is filming of unsynchronized cells.)
We have now revised this experiment and have included the combination between Mad1-EDD/QNN or -Δhelix mutant and the dimerization-deficient Mad2-R133A mutant. We show that the Mad2/Mad1 ratio at kinetochores is lower with Mad2-R133A than with wt Mad2, not only in wt Mad1, but also in Mad1 mutants (Fig 3G). This is an additional indication that dimerization of wt Mad2 is not impaired in these Mad1 mutants. We think that the consistent effect between strains supports our conclusion, despite the low cell number in each experiment. In addition, we now provide a statistical analysis of the time course data (supplementary Fig S4F). The difference in the Mad2/Mad1 ratio between wt Mad2 and Mad2-R133A is statistically significant in all three Mad1 strains, whereas there is no statistically significant difference in this ratio between the three Mad1 strains with wt Mad2.

Referee #3:

The spindle checkpoint ensures the fidelity of chromosome segregation by delaying anaphase onset until all chromosomes are properly attached to spindle microtubules. Mad1 and Mad2 are both critical downstream components of this checkpoint. Mad2 is an unusual two-state protein: the active C-Mad2 and the inactive O-Mad2. It is believed that a Mad1-C-Mad2 core complex needs to be recruited to unattached kinetochores, where it "catalyzes" the efficient conversion of cytosolic O-Mad2 to active C-Mad2.

In this manuscript, the authors show that the conserved RLK motif in the C-terminal domain (CTD) of the S. pombe Mad1 is critical for the kinetochore localization of Mad1, but is not required for the Mad1-Mad2 interaction. Mutations in the Mad1 RLK motif abolish the spindle checkpoint. Consistently, mutations of the putative RLK motif-binding region on Bub1 also cause Mad2 delocalization from kinetochores and a defective spindle checkpoint. Furthermore, a Mis12-Mad1-RLK/AAA fusion protein (which localizes to kinetochores) restores Mad2 kinetochore localization, but cannot restore the spindle checkpoint. These data indicate that the RLK motif has a checkpoint role in addition to targeting Mad1-Mad2 to kinetochores. Finally, mutations of the extremely C-terminal helix of Mad1 also abolish checkpoint signaling, without disrupting the Mad1-Mad2 interaction and their kinetochore localization. Taken together, their results suggest that Mad1 CTD has a more direct role in the
spindle checkpoint, aside from recruiting the tightly bound C-Mad2.

Overall, the manuscript contains several interesting findings. Most of the results are solid, with the few exceptions noted below. These specific points need to be addressed. In addition, the new function of Mad1 CTD has not been mechanistically characterized. The model in the end is highly speculative. This speculation needs to be toned down. If these issues can be addressed, publication in EMBO Reports is recommended.

Minor points:

(1) In Fig. 1, the Bub1 cm1 mutant had normal kinetochore localization, but severely reduced Mad2 localization on kinetochores. Does this mutant also reduce Mad1 kinetochore localization, as would be expected?

> Yes, Mad1 (new Fig 1J,K) as well as Mad2 localization (new Fig S1I) are perturbed in the *bub1-cm1* mutant, whereas localization of Bub1 (Fig 1K), Bub3 and Mad3 (new Fig S1I) is preserved.

(2) The authors state that Mad1Δhelix keeps Mad1-Mad2 binding intact. In Fig. 3F, Mad2 binding to Mad1Δhelix appeared to be weakened. The conclusions drawn from Δhelix mutant need to be revised.

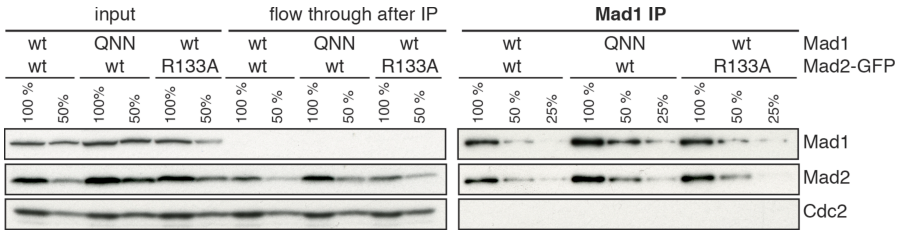
> Because loading was previously unequal, we now show another repeat of the same experiment (Fig 3F). We do not observe any strong difference in Mad2 association between wt Mad1 and Mad1 mutants. There may be slightly less Mad2 associated with Mad1-Δhelix than with wild type Mad1, but there is certainly not less Mad2 co-immunoprecipitated with Mad1-EDD/QNN. In any case, the differences (if at all existent) are small, and we also do not detect any obvious difference in quantifications of the Mad2/Mad1 ratio by microscopy (Fig 3G).
> Since we observe that taking away the C-terminal GFP-tag on Mad1-Δhelix leads to lower protein abundance (new Fig S4H; Fig 3E shows the abundance with the GFP tag present), it is possible that deletion of the C-terminal helix slightly destabilizes Mad1 and this may lead to a decay of the protein during the immunoprecipitation procedure. Since the ratio between Mad2 and Mad1 as judged by microscopy is maintained, and since we see less Mad2 recruitment in the Mad1-Δhelix mutant when Mad2-R133A rather than wt Mad2 is expressed, we think it is overall appropriate to conclude that Mad1-Δhelix (and Mad1-EDD/QNN) strongly impairs checkpoint activity without obviously impairing Mad2 recruitment or dimerization.

(3) It is stated that the Mad2 R133A mutation causes a reduction of Mad2/Mad1 ratio and Mad2 kinetochore signal (Fig3. G and H). Statistic analysis of both sets of data is required to support their statement. In addition, this statement would be further bolstered if they can show a reduced Mad2 signal in Mad1 IP.

> We have now additionally analysed the Mad2-R133A/Mad1 ratio in Mad1-EDD/QNN and Mad1-Δhelix and we have performed a pooled component test (Wu et al., Biometrics 2006), which is suited to statistically analyse time-series data (Heinrich NCB 2013). The result is shown in Fig S4E,F. The difference in the Mad2/Mad1 ratio between wt Mad2 and Mad2-R133A is statistically significant in all three Mad1 strains, whereas there is no statistically significant difference in the Mad2-wt/Mad1 ratio between the three Mad1 strains.

We are not aware of data showing that a deficiency in Mad2 dimerization can be seen in a Mad1 immunoprecipitation. (Since the turnover of the additional Mad2 is high, this pool of Mad2 may not be recovered in the IP.) To our knowledge, neither DeAntoni Curr Biol 2005 (Figure 5C) nor Vink JCB 2006 see a reduction in the Mad2-R133A/Mad1 ratio compared to the Mad2-wt/Mad1 ratio by immunoprecipitation. In our own immunoprecipitation (see below), we also do not observe any strong difference.

However, by now showing the additional combinations between Mad1 mutants and Mad2-R133A (Fig 3G), we provide additional evidence that Mad2 dimerization is intact in the Mad1 C-terminal mutants.



(4) There is an inconsistency in the phenotype of the Mad1 fragment 458-676. In Fig. S2 C and E, fragment 458-676 shows reasonable kinetochore localization, similar to WT and 306-676; however, in panel D, the kinetochore signal of the same fragment is somewhat defective. Why is this?

> We believe that this might be due to different behaviour at different temperatures.
Mad1 458-676 does enrich more strongly at kinetochores at 30 °C (supplementary Fig S2C,E) than at 16 °C (supplementary Fig S2D). Because the cytoplasmic signal is also more prominent at 30 ºC, the difference may reflect a change in overall abundance, potentially due to different protein stability at the different temperatures. Signal quantification at the kinetochore shown in supplementary Fig S2D was performed with the low exposure times that we use to preserve viability in live cell imaging. We re-imaged with longer exposure times (example pictures in Fig S2D), which then revealed a difference between Mad1 458-676 and 564-676, but indeed showed lower intensity for 458-676 compared to wt or 306-676.
Since we assay checkpoint activity at 16 ºC (the restrictive temperature for *nda3-KM311*), the checkpoint defect in the Mad1 458-676 mutant may be partly due to the overall lower level. We therefore think that we cannot draw any strong conclusions from this mutant and only stated in the text (page 5) that "… the Mad1 N-terminus… was at least partly dispensable for kinetochore localisation", which is based on the Mad1 306-676 mutant.

2nd Editorial Decision 07 January 2014

I have now received feedback from the three referees who were asked to assess your study, who as you will see below all now support publication of your study. I am thus happy to write with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once a few minor formatting issues have been addressed, as follows.

- I have noted that the length of the text is somewhat longer than we can accommodate. As you don't have many figures, I will not insist on this, but I do think that the legend to figure 4 should be shortened, as this discussion belongs in the main text.

- We now encourage the publication of original source data for the key experiments in a study - particularly for electrophoretic gels and blots, but also for graphs- with the aim of making primary data more accessible and transparent to the reader. If you agree, you would need to provide one PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figure and an Excel sheet or similar with the data behind the graphs. The files should be labeled with the appropriate figure/panel number, and the gels should have molecular weight markers; further annotation could be useful but is not essential. The source files will be published online with the article as supplementary "Source Data" files and should be uploaded when you submit your final version.

Once all remaining corrections have been attended to, you will receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt inclusion of your manuscript in our next available issue.

Many thanks for your contribution to EMBO reports.

REFEREE REPORTS:

Referee #1:

I wish to thank the authors for their thoughtful consideration of the reviewers' comments and for submitting an improved version of their manuscript. I strongly support acceptance and publication of the manuscript.

Referee #2:

The authors have done a good job here, having addressed most of the important issues raised. These have definitely led to an improved manuscript, and I am now happy to recommend that this be accepted for publication.

Referee #3:

The authors have adequately addressed my concerns. Publication is recommended.

2nd Revision - authors' response 08 January 2014

We have made the required changes and are uploading the following files:

- Article file:
    - contains title and abstract as you suggested
    - synopsis with bullet points added
    - most of legend to Figure 4 incorporated into the text
    - Kruse et al., in press, cited
- Figure files:
    - in Adobe Illustrator format
    - no changes were made

- Supplementary Information + Supplementary Table 1
    - reference format modified, otherwise no changes
- Graphic file for synopsis and thumbnail
    - in Adobe Illustrator format
- Source data:
    - 3 excel files, 3 pdf files

If anything else is required, please let me know.

3rd Editorial Decision 09 January 2014

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

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Thank you again for your contribution to EMBO reports and congratulations on a successful publication.

