A direct role of Mad1 in the spindle assembly checkpoint beyond Mad2 kinetochore recruitment

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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, please accept my apology for the time it has taken us to contact you with a full set of referee reports, given that the last one of them was considerably delayed. As you will see in the reports pasted below, although all the referees find the topic of interest, referees consider the reported advance relatively limited and all point out various issues regarding the conclusiveness and technical soundness of the dataset.

As the reports are very detailed, I will not belabor them here. Notably, it will be important to bolster the conclusiveness of your data, as the study does need to stand alone, as referee 1 points out (although part of a back-to-back submission). Referees 1 and 2 in particular raise numerous issues, all of which would need to be addressed, as would those pointed out by referee 3. Referees 1 and 2 also point out the limited novelty of the data and the need to adequately reference and discuss previous studies so the this fact is clear, and your findings adequately contextualized within the current state of the field.

Given that all referees provide constructive suggestions on how strengthen the work and make it more conclusive, I would like to give you the opportunity to revise your manuscript. As you 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 manuscript, the authors report several experiments aiming to prove that Mad1 is required for the spindle checkpoint in addition to its role as a recruiter of Mad2 to the kinetochore. As explained below, the conclusion is not novel, although clearly the work described here significantly extends previous efforts. Indeed, the authors have clearly put significant effort in the preparation of this manuscript, which deserves praise.

This manuscript was co-submitted with a manuscript from the group of Dr. S. Hauf in which similar conclusions are reached. The two manuscripts are complementary, and together they make a strong point with regard to the claim that Mad1 does not simply act as a passive carrier of Mad2. Nevertheless, for this manuscript to stand on its own leg, the authors should address some logical and technical weaknesses, as detailed below.

Specific points

Subjectively, I find the approach to citing previous literature in this manuscript somewhat subjective. Most notably, the authors did not cite the Brady and Hardwick Current Biology paper in which it is reported that the C-terminal region of Mad1 is required for an interaction with Bub1 and for the role of Mad1 in the checkpoint. To the extent that this manuscript brings relatively little additional mechanistic insight into the process, there is a concern on the novelty of the work, and selective citing needs not obscure this.

The main assumption to justify the existence of another role of Mad1 in the checkpoint is stated on page 7, where the authors argue: "That Mad1 was still required for a SAC arrest despite the continuous kinetochore targeting of C-Mad2 suggested additional critical roles of Mad1 in the SAC beyond Mad2 kinetochore recruitment." They repeat the concept on page 9, where they write: "...reaffirming that failure in inducing an arrest is not due to lack of C-Mad2 binding". The demonstration that the C-Mad2 mutant can in principle bypass the specific requirement of Mad1 in stabilizing C-Mad2 at the kinetochore, allowing exposing another role of Mad1, is essential for this manuscript to stand up. If C-Mad2 does not complement the depletion of Mad1, this does not imply at all that Mad1 does something else in the checkpoint. It simply means that C-Mad2 does not do what it is supposed to do when Mad1 is not there. Mad1 might remain a necessary holder of Mad2 even when the available Mad2 is the C-Mad2 mutant, and in addition to any possible additional function. In my view this is a major logical weakness of the paper, when considered in isolation from the paper from Hauf and co-workers.

To prove their point, the authors should show instead that the checkpoint defect caused by the removal of the Mad2 binding site from Mad1 is bypassed when C-Mad2 is expressed. If this were the case (I predict it is not) then the authors could go ahead and test their mutations in the C-terminal region of Mad1 and demonstrate an additional and independent role of Mad1 in the checkpoint. If this were not the case, however, the conclusion, that the mutations identified by the authors do
anything different than stabilizing the structure and function of C-Mad2 would remain undemonstrated.

To be sure, there are multiple intrinsic reasons why it is unlikely that C-Mad2 complements the loss of the Mad2-binding domain of Mad1, as well as the important extrinsic reasons that the L13A mutant is a poor C-Mad2 mutant, as shown rigorously by Mapelli et al. Cell 2007. L13Q is considerably more penetrant, and should have been used instead. The antibody approach used by the authors to demonstrate that there is enough C-Mad2 at kinetochores is not ideal, not least because the antibody itself is likely to contribute to the stabilization of the C-Mad2 conformation when it binds to Mad2. Among the intrinsic reasons why C-Mad2 at kinetochores is unlikely to complement the loss of Mad1 is also the consideration that Mad1 dimerization might by itself contribute to Mad2 function.

As a minimum, it would have made more sense to run the whole series of mutants in Figure 4 with the C-Mad2 mutant to demonstrate convincingly (or rule out) that what is missing is only the sought-for additional function of Mad1 and not some significant residual requirement of Mad1 in C-Mad2 stabilization. As presented, the approach chosen here falls short of showing the central assumption of the manuscript convincingly.

Minor points

Introduction
Second paragraph: It is not clear what the authors mean by "extreme" conformations. The authors could replace "extreme" with "at least two"

First paragraph: Please cut sentences when they get too long. For instance, split the sentence ...BubR1-Bub3, following..." by adding a point after Bub3.

Page 9, first paragraph: "...all the cells analysed...". It is not clear which cells are the authors referring to.

Referee #2:

The authors employ Ndc80-tethering experiments to recruit Mad2 to kinetochores independently of Mad1. This leads to SAC arrest, that is Mad1 dependent. Tethering of mutant alleles (O-Mad2 and non-dimerizing R133A Mad2) did not produce an arrest. A series of Mad1 mutants are tested (and tethered themselves), and it is concluded that Mad1 has a direct role in the SAC beyond Mad2 recruitment.

Figure 1: the labelling needs to be clearer. eg. Fig1B: apart from KT-Mad2 the others are NOT Ndc80 fusions. The schematic models don't help much, partly because their legend is too far away.

Figure 1D: why are KT-Mad2and KT-C-Mad2 so different in the pull-down? Why doesn't KT-Mad2 pull down Cdc20 and p31?

Figure 2: overexpression of Mad2 was reported to perturb MT dynamics in vertebrate cells (Kabeche and Compton, Curr Biol 2012). It could stabilise MTs, lead to lagging chromosomes, and perturb Aurora B localisation. Could there be perturbation of MT dynamics here, when Ndc80-Mad2 is expressed? Presumably this competes with endogenous Ndc80 for interactions within the KMN network and binding to microtubules. The authors need to look more carefully at these kinetochores, particularly as there does appear to be subtle changes in inter KT distance. What about intra-KT stretch? We should be shown, on an anti-Mad2 western, how the levels of endogenous Mad2 and the Mad2-Ndc80 fusions compare.

Figure 3: the dependency on Mad1, BubR1, Mps1 etc. is used to argue that targeted Mad2 induces a genuine SAC arrest. Does it require Bub1 and Aurora kinase activity too?

Figure 4: in Fig 4D why is D1-500 not recruited to KTs - it can bind Mad2 (Fig. 4B). Have the
authors tried to get the C-terminus of Mad1 to bind to another protein e.g. in biochemical
purifications or two-hybrid assays?

p10. Neither this study, nor the Hauf study, have identified the new Mad1 function that is dependent
on the Mad1 C-terminus. Thus it is premature to argue that this function is conserved. Though that
seems likely, it is also possible that the C-terminus of Mad1 performs different functions in fission
yeast to human cells (both functions being important for the SAC).

Both studies are interesting, and of high technical proficiency. However, 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. Note, 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). It is
however a nice study, and will serve to remind the field of the importance of Mad1.

Referee #3:

The spindle checkpoint delays anaphase onset until all chromosomes are properly attached to
spindle microtubules, thus ensuring faithful chromosome segregation. Mad1 and Mad2 are key
players of this checkpoint. Spindle checkpoint activation requires the kinetochore recruitment of the
Mad1-C-Mad2 core complex, which then promotes the conversion of inactive cytosolic O-Mad2
into active C-Mad2. Studies on the checkpoint function of Mad1 in mammalian cells have been
hampered by the fact that siRNA-mediated Mad1 depletion is insufficient to turn off the checkpoint
in the presence of high concentrations of spindle poisons.

In this manuscript, the authors use a gain-of-function assay to study the role of the conserved C-
terminal domain (CTD) of Mad1 in human cells. They show that forced targeting of Mad2 to
kinetochores causes a prolonged mitotic arrest, which is dependent on many checkpoint
components, including the endogenous Mad1 and Mad2. Using this system (and a system with
forced kinetochore targeting of Mad1 developed earlier by Kapoor and colleagues), they show that
the Mad1 CTD has a novel checkpoint role, in addition to targeting the tightly bound C-Mad2 to
kinetochores. Although the molecular basis for this new function is not established, the
demonstration that Mad1 has a novel checkpoint function is significant, and would inspire future
work to delineate the mechanism. The data generally support their conclusions, with some
exceptions noted below. Publication on EMBO Reports is recommended, if the authors could
address the following specific points.

Minor points:
(1) The authors should mention the caveat that the forced targeting of Mad1 or Mad2 to
kinetochores is an artificial way to activate the spindle checkpoint. The definitive experiment to
demonstrate a requirement for the Mad1 CTD in the spindle checkpoint is to deplete endogenous
Mad1 and complement with Mad1 CTD mutants. If this could not be done, there is a formal
possibility that the endogenous Mad1 protein does not use the same mechanism to activate Mad2.
This caveat needs to be discussed.

(2) Proper statistic analysis is needed to support the statement that Mad1 depletion causes decreased
C-Mad2 intensity in KT-Mad2 cells, but not KT-C-Mad2 cells.

(3) References that initially reported the Mad2 L13A and V193N mutants need to be cited.

(4) Fig. 1D doesn't show convincing binding between KT-Mad2 and Cdc20. A longer exposure of
the IP-western might be needed.

(5) In page 12, second paragraph "IMMUNOFLUORESCENCE", 6th line, "Glycin" should be
"Glycine".

(6) In page 12, 3rd paragraph "TISSUE CULTURE", are the units of Nocodazole and Taxol
concentrations ng/ml, instead of "ng/µl"?
(7) In Fig. 1B and C, Western blot of different Mad2 constructs is needed to rule out the possibility that distinct phenotypes are caused by different expression levels. Similarly, the levels of ectopic Mad1 expression need to be compared to the endogenous Mad1.

(8) In Fig. 4A, it is better to remove TFP tag from the construct map. Otherwise, the numbering could be somewhat misleading.

(9) In Fig. 4D, bottom panel, the Mad1 construct is labeled as "1-538", which might be a typo.

1st Revision - authors' response 20 December 2013

Referee #1:

In this manuscript, the authors report several experiments aiming to prove that Mad1 is required for the spindle checkpoint in addition to its role as a recruiter of Mad2 to the kinetochore. As explained below, the conclusion is not novel, although clearly the work described here significantly extends previous efforts. Indeed, the authors have clearly put significant effort in the preparation of this manuscript, which deserves praise.

We thank the reviewer for the positive comments and input to the manuscript. However, we disagree with the reviewer that convincing experimental data exists to show that Mad1 has additional roles in the SAC as no prior work has been able to uncouple Mad1 kinetochore localization and SAC activities. The Mad1 RLK mutant identified in the Brady and Hardwick study does not localize to kinetochores and this is most likely the cause of the observed SAC defect.

This manuscript was co-submitted with a manuscript from the group of Dr. S. Hauf in which similar conclusions are reached. The two manuscripts are complementary, and together they make a strong point with regard to the claim that Mad1 does not simply act as a passive carrier of Mad2. Nevertheless, for this manuscript to stand on its own leg, the authors should address some logical and technical weaknesses, as detailed below.

Specific points

Subjectively, I find the approach to citing previous literature in this manuscript somewhat subjective. Most notably, the authors did not cite the Brady and Hardwick Current Biology paper in which it is reported that the C-terminal region of Mad1 is required for an interaction with Bub1 and for the role of Mad1 in the checkpoint. To the extent that this manuscript brings relatively little additional mechanistic insight into the process, there is a concern on the novelty of the work, and selective citing needs not obscure this.

We agree with the reviewer on this point and have now discussed the important Brady and Hardwick paper in relation to our work. It is important to point out that the RLK Mad1 mutant used in that study does not localize to kinetochores (see for example Kim et al 2012). Therefore, a conclusion on additional roles of Mad1 in the SAC cannot, in our view, be firmly concluded from this mutant. The SAC defect of the RLK Mad1 mutant could simply be due to its failure in localizing to kinetochores. In our work by directly tethering Mad1 we circumvent this problem allowing us to show that Mad1 has additional roles in the SAC besides recruiting Mad2 to kinetochores. Whether the role of the C-terminus of human Mad1 is to bind Bub1 to assemble a larger checkpoint complex is unclear to us as we have not been able to detect
Bub1 in deep mass spec screens of purified endogenous and exogenous Mad1 from nocodazole arrested cells. Furthermore extensive yeast two-hybrid screens with Mad1 or its C-terminal domain have failed to identify Bub1. This is in agreement with work from the Luo and Yu labs that have also failed to detect an interaction between Mad1 and Bub1 (see discussion in Kim et al 2012). It could be that the interaction between the two proteins is very weak in human cells compared to budding yeast. We now discuss these possibilities in the revised manuscript.

The main assumption to justify the existence of another role of Mad1 in the checkpoint is stated on page 7, where the authors argue: "That Mad1 was still required for a SAC arrest despite the continuous kinetochore targeting of CMad2 suggested additional critical roles of Mad1 in the SAC beyond Mad2 kinetochore recruitment." They repeat the concept on page 9, where they write: "...reaffirming that failure in inducing an arrest is not due to lack of CMad2 binding". The demonstration that the C-Mad2 mutant can in principle bypass the specific requirement of Mad1 in stabilizing C-Mad2 at the kinetochore, allowing exposing another role of Mad1, is essential for this manuscript to stand up.

We disagree with the reviewer on this point. Our extensive efforts with generating a closed specific Mad2 antibody to address the level of C-Mad2 in the different experimental settings have shown that in KT-C-Mad2 the level of closed Mad2 remains constant despite removal of Mad1 (Figure 3E-F). We have also strengthened this conclusion by staining for p31 in KT-C-Mad2 expressing cells and shown that the levels of p31 are not affected by Mad1 depletion (new supplemental Figure S3A).

Furthermore, using this antibody we have analyzed the ability of different KTMad1 constructs to recruit C-Mad2 to kinetochores and find that the deletion of the C-terminal domain of Mad1 does not affect the kinetochore levels of CMad2 (Supplemental Figure S2 in original submission). The structure of the Mad1 (residues 485-584)-Mad2 complex (Sironi et al 2002) clearly shows that this very small fragment of Mad1 is sufficient to lock Mad2 in its closed conformation. This means that the “holder” function of Mad1 is maintained in this fragment and likely in the short sequence that binds Mad2 (similar to what is observed with the Mad2-Cdc20 interaction in Barfords MCC structure (Chao et al 2012) and the MBP1 peptide used in Luo et al 2002 and Mapelli et al 2007). Accordingly, in all the KT-Mad1 constructs where we have not mutated the Mad2 binding site the “holder” function of Mad1 for Mad2 are in place yet for instance KT-Mad1 1-639, KT-Mad1 F712A/R714A and KT-Mad1 E710A/F712A is severely impaired in SAC signaling compared to KT-Mad1 clearly revealing additional roles for Mad1 in the SAC beyond acting as a holder for Mad2.

It is possible that the globular C-terminal domain of Mad1 interacts weakly with Mad2 but extensive biochemical efforts using recombinant proteins have failed to detect any interaction even at high protein concentrations. Therefore we do not favor that the globular domain of Mad1 interacts with Mad2.

If C-Mad2 does not complement the depletion of Mad1, this does not imply at all that Mad1 does something else in the checkpoint. It simply means that CMad2 does not do what it is supposed to do when Mad1 is not there.

This it was we wanted to find out with all our experiments as the “template model” does not propose other functions of Mad1 than placing C-Mad2 at kinetochores. The work from Simonetta et al 2007 compared the ability of CMad2 and Mad1 (485-718)-Mad2 complex to stimulate the binding of Mad2 wt to a Cdc20 peptide and found no difference. This would suggest that if the only function of Mad1 were to place C-Mad2 at kinetochores to provide a dimerization surface for O-Mad2 then placing C-Mad2 at kinetochores would bypass the requirement of Mad1. This is clearly not what we see.
Mad1 might remain a necessary holder of Mad2 even when the available Mad2 is the C-Mad2 mutant, and in addition to any possible additional function. In my view this is a major logical weakness of the paper, when considered in isolation from the paper from Hauf and co-workers.

Although our Mad2 tethering experiments is one approach to probe for additional functions of Mad1 our extensive efforts to map precisely the specific regions and residues of Mad1 for this function is equally important. Our ability to identify deletions and mutations in Mad1 that clearly have maintained their ability to act as “holder” of Mad2 yet are defective in the SAC despite being tethered to the kinetochore is a key and strong point of our work.

To prove their point, the authors should show instead that the checkpoint defect caused by the removal of the Mad2 binding site from Mad1 is bypassed when C-Mad2 is expressed. If this were the case (I predict it is not) then the authors could go ahead and test their mutations in the C-terminal region of Mad1 and demonstrate an additional and independent role of Mad1 in the checkpoint. If this were not the case, however, the conclusion, that the mutations identified by the authors do anything different than stabilizing the structure and function of C-Mad2 would remain undemonstrated.

Expression of soluble C-Mad2 at much higher levels than KT-C-Mad2 hardly induces any mitotic delay (Figure 1B) suggesting that it is critical that C-Mad2 is positioned at kinetochores for generating a SAC arrest. Our work shows that in addition to having C-Mad2 at kinetochores additional roles are played by Mad1 and we believe that both activities have to occur at the kinetochore for a functional SAC.

To be sure, there are multiple intrinsic reasons why it is unlikely that C-Mad2 complements the loss of the Mad2-binding domain of Mad1, as well as the important extrinsic reasons that the L13A mutant is a poor C-Mad2 mutant, as shown rigorously by Mapelli et al. Cell 2007. L13Q is considerably more penetrant, and should have been used instead.

The reason why we did not originally use the L13Q mutant of Mad2 is that work from Stephen Taylor has revealed that this mutant behaves “strangely” in vivo in that it does not readily bind Cdc20 or Mad1 (Figure 7 in Westhorpe et al 2011). Furthermore, in the paper from Mapelli et al 2007 it was concluded that: “Strikingly, Mad2 L13A and Mad2 L13Q preferred the CMad2 conformation, recapitulating the effects of the ‘topological’ mutants Mad2 DN15 and Mad2 DN15-LL. Like Mad2 DN15 and Mad2 DN15-LL, Mad2 L13A and Mad2 L13Q retained the ability to bind GST-Cdc20111–138”. Also, The Mad2 L13A and Mad2 L13Q mutants behaved identically in Resource Q experiments in that work (Figure 3C in Mapelli et al 2007). Furthermore, the presence of “large” tags on the N-terminus of Mad2 favors the closed conformation of Mad2 (see for example Tipton et al 2011). We have now included our own Resource Q analysis of Mad2 V193N and Mad2 L13A and confirmed that Mad2 L13A is exclusively in the closed conformation (New Figure S1B). Furthermore, we have as suggested by the reviewer, analyzed whether KT-Mad2 L13Q is dependent on Mad1 for a metaphase checkpoint arrest and indeed this is the case (New Figure S1D).

The antibody approach used by the authors to demonstrate that there is enough C-Mad2 at kinetochores is not ideal, not least because the antibody itself is likely to contribute to the stabilization of the C-Mad2 conformation when it binds to Mad2.

This is difficult to test but our experiments in Figure 3 shows that the antibody can report on the level of C-Mad2 at kinetochores. The fixation of cells with formaldehyde likely also crosslinks Mad2 preventing the C-Mad2 antibody
from inducing major conformational changes. We have now also in completely parallel experiments measured the level of C-Mad2 in KT-Mad1 and KT-CMAd2 to see if there was a large difference, which is not the case (new Figure S3B). Furthermore as discussed above we have included p31 staining as an alternative approach for measuring C-Mad2 levels on the kinetochore (new Figure S3A).

Among the intrinsic reasons why C-Mad2 at kinetochores is unlikely to complement the loss of Mad1 is also the consideration that Mad1 dimerization might by itself contribute to Mad2 function.

Mad1 dimerization is potentially important but the mutations in the globular domain (Mad1 F712A/R714A and Mad1 E710A/F712A) are not involved in dimerization (Kim et al 2012) yet are checkpoint defective.

As a minimum, it would have made more sense to run the whole series of mutants in Figure 4 with the C-Mad2 mutant to demonstrate convincingly (or rule out) that what is missing is only the sought-for additional function of Mad1 and not some significant residual requirement of Mad1 in C-Mad2 stabilization. As presented, the approach chosen here falls short of showing the central assumption of the manuscript convincingly.

We have now, as suggested, analyzed in the KT-C-Mad2 cell line the key requirement for the Mad1 C-terminal globular domain in inducing an arrest and observe the same requirement as observed for KT-Mad2 (New supplemental Figure S3C).

Minor points

Introduction
Second paragraph: It is not clear what the authors mean by "extreme" conformations. The authors could replace "extreme" with "at least two"

Done

First paragraph: Please cut sentences when they get too long. For instance, split the sentence ...BubR1-Bub3, following..." by adding a point after Bub3.

We have aimed at doing this in the revised manuscript.

Page 9, first paragraph: "...all the cells analysed...". It is not clear which cells are the authors referring to.

This is clarified now.

Referee #2:

The authors employ Ndc80-tethering experiments to recruit Mad2 to kinetochores independently of Mad1. This leads to SAC arrest, that is Mad1 dependent. Tethering of mutant alleles (O-Mad2 and non-dimerizing R133A Mad2) did not produce an arrest. A series of Mad1 mutants are tested (and tethered themselves), and it is concluded that Mad1 has a direct role in the SAC beyond Mad2 recruitment.

Figure 1: the labelling needs to be clearer. eg. Fig1B: apart from KT-Mad2 the others are NOT Ndc80 fusions. The schematic models don't help much, partly because their legend is too far away.

We have modified the labeling to make it more clear and also moved the schematic up to bring it closer to the relevant figures.
Fig 1D: why are KT-Mad2 and KT-C-Mad2 so different in the pull-down? Why doesn't KT-Mad2 pull down Cdc20 and p31?

The KT-Mad2 and KT-C-Mad2 appears to bind very similar levels of Mad1 yet the KT-C-Mad2 is more stably bound to Cdc20 and p31. We believe that the reason for the difference is that the Mad2 L13A mutation stabilizes the Cdc20-Mad2 interaction since it prevents the conversion of closed Mad2 to open Mad2. The dissociation of Mad2 from Cdc20 is stimulated by p31 (Westhorpe et al 2011), which likely explains the increase in p31 levels in KT-C-Mad2. In contrast the Mad1-Mad2 interaction is very stable and appears not to be regulated by p31 and this is likely why there is no difference in Mad1 binding.

Figure 2: overexpression of Mad2 was reported to perturb MT dynamics in vertebrate cells (Kabeche and Compton, Curr Biol 2012). It could stabilise MTs, lead to lagging chromosomes, and perturb Aurora B localisation. Could there be perturbation of MT dynamics here, when Ndc80-Mad2 is expressed? Presumably this competes with endogenous Ndc80 for interactions within the KMN network and binding to microtubules. The authors need to look more carefully at these kinetochores, particularly as there does appear to be subtle changes in inter KT distance. What about intra-KT stretch? We should be shown, on an anti-Mad2 western, how the levels of endogenous Mad2 and the Mad2-Ndc80 fusions compare.

We have included blots showing the level of KT-Mad2 versus endogenous Mad2 (New Figure S1A), which shows that the levels of KT-Mad2 are very low compared to endogenous Mad2. In the Compton study approximately 5-10 fold higher levels of mCherry-Mad2 is expressed compared to endogenous Mad2 and a rough estimate would be that this is 500-1000 times more than what we are expressing in the KT-Mad2 cell lines. The level of KT-Mad2 (Ndc80-Venus-Mad2) compared to endogenous Ndc80 is approximately 10% and our data would suggest that at this level of expression kinetochore function is not perturbed. We have now analyzed the time from NEDB-Metaphase in Ndc80-Venus and KT-Mad2 cell lines and observe no major differences (New Figure 2C).

The reason for the increase in KT distance at least for KT-Mad2 and KT-CMAD2 could be that cells arrest for a prolonged time with a metaphase plate until they undergo cohesion fatigue. In the original characterization of cohesion fatigue it was reported that KT distances increased upon prolonging metaphase (see for example Stevens et al 2011). We are unfortunately not in a position to measure intra-KT stretch.

Figure 3: the dependency on Mad1, BubR1, Mps1 etc. is used to argue that targeted Mad2 induces a genuine SAC arrest. Does it require Bub1 and Aurora kinase activity too?

We have tested this now and the arrest requires Bub1 (New Figure 3D). The effect is not as big as with Mad2 and BubR1 RNAi but this likely reflects that very little Bub1 activity is required for the SAC (see for example discussion and data in Meraldi et al 2005 where they show that 2-5% of Bub1 at kinetochores is sufficient to maintain the SAC). We have also inhibited Aurora B using ZM447439 but these experiments are difficult to score precisely as this inhibitor also prevents cytokinesis. We have, therefore, not included it in the manuscript.

Figure 4: in Fig 4D why is D1-500 not recruited to KTs - it can bind Mad2 (Fig. 4B).

We have no good explanation for why Mad1 del 1-500 does not get recruited to kinetochores in KT-Mad2 cells.
Have the authors tried to get the C-terminus of Mad1 to bind to another protein eg. in biochemical purifications or two-hybrid assays?

Yes extensively. Both mass spectrometry screens of purified Mad1 (endogenously and exogenously) from nocodazole arrested cells as well as extensive yeast two-hybrid screens with full length Mad1 and the C-terminal globular domain of Mad1 has failed to identify meaningful binding partners. We have checked whether the globular C-terminal domain binds Mad2 and this is not the case at least using purified proteins from E.Coli.

p10. Neither this study, nor the Hauf study, have identified the new Mad1 function that is dependent on the Mad1 C-terminus. Thus it is premature to argue that this function is conserved. Though that seems likely, it is also possible that the C-terminus of Mad1 performs different functions in fission yeast to human cells (both functions being important for the SAC).

Agree and we have changed the wording.

Both studies are interesting, and of high technical proficiency. However, 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. Note, 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). It is however a nice study, and will serve to remind the field of the importance of Mad1.

We thank the reviewer for these positive and constructive comments. We agree that previous studies have indicated additional mitotic functions of Mad1 but not additional functions in the SAC. Mad1 has also been implicated in nucleocytoplasmic transport in budding yeast (see for example Louk et al 2002). This could account for the more severe loss of function phenotype of Mad1 compared to Mad2 observed in genetic experiments.

Referee #3:

The spindle checkpoint delays anaphase onset until all chromosomes are properly attached to spindle microtubules, thus ensuring faithful chromosome segregation. Mad1 and Mad2 are key players of this checkpoint. Spindle checkpoint activation requires the kinetochore recruitment of the Mad1-CMad2 core complex, which then promotes the conversion of inactive cytosolic O-Mad2 into active C-Mad2. Studies on the checkpoint function of Mad1 in mammalian cells have been hampered by the fact that siRNA-mediated Mad1 depletion is insufficient to turn off the checkpoint in the presence of high concentrations of spindle poisons.

In this manuscript, the authors use a gain-of-function assay to study the role of the conserved C-terminal domain (CTD) of Mad1 in human cells. They show that forced targeting of Mad2 to kinetochores causes a prolonged mitotic arrest, which is dependent on many checkpoint components, including the endogenous Mad1 and Mad2. Using this system (and a system with forced kinetochore targeting of Mad1 developed earlier by Kapoor and colleagues), they show that the Mad1 CTD has a novel checkpoint role, in addition to targeting the tightly bound C-Mad2 to kinetochores. Although the molecular basis for this new function is not established, the demonstration that Mad1 has a novel checkpoint function is significant, and would inspire future work to delineate the mechanism. The data generally support their conclusions, with some exceptions noted below. Publication on EMBO Reports is recommended, if the authors could address the following specific points.
We thank the reviewer for the positive comments and suggestions to improve the work.

Minor points:
(1) The authors should mention the caveat that the forced targeting of Mad1 or Mad2 to kinetochores is an artificial way to activate the spindle checkpoint. The definitive experiment to demonstrate a requirement for the Mad1 CTD in the spindle checkpoint is to deplete endogenous Mad1 and complement with Mad1 CTD mutants. If this could not be done, there is a formal possibility that the endogenous Mad1 protein does not use the same mechanism to activate Mad2. This caveat needs to be discussed.

We have done this experiment now comparing untethered FL Mad1 and Mad1 F712A/F714A and this revealed that these point mutants render Mad1 inactive (New Figure S4C). With our protocol the depletion of Mad1 is so effective that we actually see that cells progress faster through an unperturbed mitosis similar to what is described in the work from Erich Nigg and Anna Santamaria (Fava et al 2011) when they injected Mad1 antibodies. This can be rescued by FL Mad1 but not Mad1 F712A/R714A.

We were unable to test Mad1 E710A/F712A as the stable cell lines generated for this construct expressed at very low levels.

(2) Proper statistic analysis is needed to support the statement that Mad1 depletion causes decreased C-Mad2 intensity in KT-Mad2 cells, but not KT-CMad2 cells.

We have added this to the figure.

(3) References that initially reported the Mad2 L13A and V193N mutants need to be cited.

We have added the Mapelli et al 2007 paper for the Mad2 mutants. We are aware that Mapelli et al 2006 also describe Mad2 L13A but a more careful analysis is provided in the 2007 paper.

(4) Fig. 1D doesn't show convincing binding between KT-Mad2 and Cdc20. A longer exposure of the IP-western might be needed.

KT-Mad2 does bind Cdc20 and p31. This is the longest exposure we had.

(5) In page 12, second paragraph "IMMUNOFLUORESCENCE", 6th line, "Glycin" should be "Glycine".

Corrected

(6) In page 12, 3rd paragraph "TISSUE CULTURE", are the units of Nocodazole and Taxol concentrations ng/ml, instead of "ng/µl"?

Yes our mistake it should be ng/ml and corrected.

(7) In Fig. 1B and C. Western blot of different Mad2 constructs is needed to rule out the possibility that distinct phenotypes are caused by different expression levels. Similarly, the levels of ectopic Mad1 expression need to be compared to the endogenous Mad1.

We have included a blot comparing the levels of KT-Mad2 and soluble Venus-Mad2 (New Figure S1C). This shows that the soluble Venus Mad2 proteins are expressed at an at least 10 fold higher level than the KT-Mad2 proteins.
We do not have stable cell lines of KT-Mad1 as this was done by transient transfection but from analysis of the fluorescence intensity from the time-lapse experiments the expression level of KT-Mad1 is similar to that of KT-Mad2.

(8) In Fig. 4A, it is better to remove TFP tag from the construct map. Otherwise, the numbering could be somewhat misleading.

We have removed this.

(9) In Fig. 4D, bottom panel, the Mad1 construct is labeled as "1-538", which might be a typo.

Yes it should be 1-598 and is now corrected.

I have now received feedback from the three referees who were asked to assess your study. As you will see, referees 2 and 3 are now positive about the study, and referee 1 - although still somewhat critical - would support publication. 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.

- There are a few missing details in the figure legends regarding the analyses performed. In figures 2B and 3F, please indicate the P values that were considered significant (*, **, ***). Do the red bars in figure 4 indicate the median? Please state this. The supplementary figure legends lack many details of the nature of the average (mean, median), error bars, P values, statistical test applied etc. Please go through these in detail and provide all necessary information; they should be as comprehensive as the legends to the main figures.

- 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 agree with the authors that a possible reason why the RLK mutant of Mad1 originally discussed by Brady and Hardwick is checkpoint defective is that it does not localize to kinetochores. But Hardwick and co-workers also showed that the mutation of the RLK motif prevented an interaction...
of Mad1 with another checkpoint protein, Bub1, which is possibly a sufficient justification for a checkpoint defect. As it seems clear from reading their rebuttal that the authors were aware of the existence of the Brady and Hardwick paper, their failure to cite it in the first version of the manuscript was not unintentional, which is disturbing to this reviewer. I write this here for future reference.

I already clarified in my review why I believe that, in the absence of the fundamental control testing whether the expression of C-Mad2 complements the expression of a form of Mad1 mutated in the Mad2-binding motif, the papers suffers from an irreparable logical breach. Repeated briefly, my point is that in the absence of such control, it is impossible to discern whether the other Mad1 mutants discussed by the authors have to do with some unknown additional aspect of the interaction of Mad1 with Mad2, or rather with some other function of Mad1 in the checkpoint, as invoked by the authors. (Incidentally, the authors' claim that the C-terminal domain of Mad1 is unlikely to interact with Mad2 is almost certainly wrong. Intra-molecular interactions usually have KDs in the millimolar range, a concentration at which the authors are unlikely to have carried out their tests.)

Instead of presenting the obvious and simple control that I had requested in the revised version of the manuscript (and which I am quite confident the authors have performed with disappointing - for them - results), the authors use their rebuttal letter to explain why they don't think this is a serious objection, additionally supported by incorrect interpretations of other people's experiments. All this annoys me, I have to confess. I personally hold the view that constructive, however painful, peer reviewing is meant to communicate to authors that there are problems with their work. But if authors are reluctant to accept the reviewers' views, and prefer to withhold essential controls in support of their claims, eventually they should be entitled to publish their work. Provided editors allow it, of course, as I hope it will be the case. I.e. I have no intention to veto the publication of this manuscript. With my reviews available via the "transparent review" process, it will be possible for readers to access a critical point of view on this manuscript and judge for themselves.

Referee #2:
The authors have done a good job here and have adequately addressed (or argued against) my criticisms and suggestions.

In combination with the revised manuscript from Silke Hauf and colleagues, this study makes a strong case for Mad1 having multiple spindle assembly checkpoint functions (not just being required to recruit and present Mad2 at kinetochores).

I am now happy to recommend its publication in EMBO Reports.

Referee #3:
The authors have adequately addressed my concerns. The addition of data on Mad1-depletion-rescue greatly strengthens the paper. I also found the authors' responses to other reviewers reasonable. I recommend the publication of this manuscript in EMBO Reports.

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It was completely unintentional that we did not cite this paper. We were focused on discussing the “template model” in the original submission and therefore forgot to also discuss the budding yeast Mad1-Bub1 interaction. We do not see in any way that discussing the Brady and Hardwick work questions our work and therefore are happy to discuss it and cite it. We are grateful that the reviewer pointed out that it should be discussed. It should be pointed out that it was not shown that the Bub1-Mad1 interaction described in budding yeast is direct. Potentially it could be bridged by another protein(s) or if the affinity of Bub1 and Mad1 for the kinetochore is very strong in budding yeast then the SAC induced kinetochore localization of these proteins could explain the results of the paper.

I already clarified in my review why I believe that, in the absence of the fundamental control testing whether the expression of C-Mad2 complements the expression of a form of Mad1 mutated in the Mad2-binding motif, the papers suffers from an irreparable logical breach. Repeated briefly, my point is that in the absence of such control, it is impossible to discern whether the other Mad1 mutants discussed by the authors have to do with some unknown additional aspect of the interaction of Mad1 with Mad2, or rather with some other function of Mad1 in the checkpoint, as invoked by the authors. (Incidentally, the authors' claim that the C-terminal domain of Mad1 is unlikely to interact with Mad2 is almost certainly wrong. Intra-molecular interactions usually have KDs in the millimolar range, a concentration at which the authors are unlikely to have carried out their tests.)

The highest concentrations we have tested in isothermal titration calorimetric experiments between recombinant Mad2 forms and recombinant Mad1 (639-718) is 0.4 mM and in analytical ultracentrifugation experiments we used 20 micromolar. At these concentrations we have failed to detect any binding but we agree with the reviewer that in the context of the Mad1-Mad2 complex there could be an intra-molecular interaction between the C-terminal domain of Mad1 and Mad2. Whether an underlying intra-molecular interaction between the C-terminal domain and Mad2 is “the mechanism” of the C-terminal domain is possible but this would still be novel and interesting as the template model poses C-Mad2 as the active molecule. We did try and investigate a possible role of the C-terminal domain of Mad1 in Mad2 turnover at kinetochores. We performed FRAP experiments of TFP-O-Mad2 in KT-Mad1 and KT-Mad1 1-598 arrested at metaphase and in both situations we see identical recovery rates (half time of recovery 4-5 seconds (rates in agreement with other measured rates in vivo and in vitro (see for instance Vink et al 2006). Thus all our attempts to see if the C-terminal domain affect Mad2 biology have been negative so far.

Instead of presenting the obvious and simple control that I had requested in the revised version of the manuscript (and which I am quite confident the authors have performed with disappointing - for them - results)

We have not performed this experiment. We are quite sure that tethering Mad1 at kinetochores with its Mad2 binding site mutated and then providing soluble closed Mad2 would not result in a metaphase arrest. A negative result would not prove us wrong but instead suggest that two functions of Mad1 have to occur (and potentially integrate) at the kinetochore.
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3rd Editorial Decision 09 January 2014

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

, the authors use their rebuttal letter to explain why they don’t think this is a serious objection, additionally supported by incorrect interpretations of other people’s experiments.

We are not aware of any incorrect interpretations of other people’s results and would have preferred a more detailed description of this from the reviewer so we could take this into account in the future.

All this annoys me, I have to confess. I personally hold the view that constructive, however painful, peer reviewing is meant to communicate to authors that there are problems with their work. But if authors are reluctant to accept the reviewers’ views, and prefer to withhold essential controls

We certainly share the reviewer’s opinions on the peer reviewing process. However, we have not withheld any information or experiments we have done and have either included them in the manuscript or discussed them in the pointto-point responses during the review process. We have also conducted several of the experiments that the reviewer asked for.

in support of their claims, eventually they should be entitled to publish their work. Provided editors allow it, of course, as I hope it will be the case. I.e. I have no intention to veto the publication of this manuscript. With my reviews available via the “transparent review” process, it will be possible for readers to access a critical point of view on this manuscript and judge for themselves.

We thank the reviewer for recommending the manuscripts publication in EMBO Reports.

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The authors have done a good job here and have adequately addressed (or argued against) my criticisms and suggestions.

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We thank the reviewer for the positive comments.

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We thank the reviewer for the positive comments.
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Thank you again for your contribution to EMBO reports and congratulations on a successful publication.