JCB

Mps1-mediated release of Mad1 from nuclear pores ensures the fidelity of chromosome segregation

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Re: JCB manuscript #201906039

Dr. Carlos Conde
IBMC, Instituto de Biologia Molecular e Celular, Universidade do Porto
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Portugal

Dear Dr. Conde,

Thank you for submitting your manuscript entitled "Mps1-mediated release of Mad1 from nuclear pores ensures the fidelity of chromosome segregation". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

The main issue to address for resubmission is the apparent discrepancy between these results as compared to those reported by Rodriguez-Bravo et al. (Cell, 2014). One possibility is the difference at multiple levels between Drosophila and human cells - a point raised by all reviewers. We would recommend checking if the Mtor phosphomutant affects MCC levels as this should provide some clarity into this issue. Regardless, there may still be residual Mad1 at NPCs that is sufficient to generate SAC function in mitosis. In addition to these points, a more thorough discussion of the differences (as pointed out by reviewers) between Mtor in flies and TPR in human cells should be included as well.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

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Text limits: Character count for a Report is < 20,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

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Our typical timeframe for revisions is three months; if submitted within this timeframe, novelty will not be reassessed at the final decision. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Timothy Yen, Ph.D.
Monitoring Editor

Marie Anne O'Donnell, Ph.D.
Scientific Editor

Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

Osswald and Cunha-Silva present a series of compelling studies examining the regulation of the Mad1-Megator interface by Mps1 kinase in this generally well-written manuscript. The authors identified a region of Megator that directly binds Mad1 in vitro and mapped four Mps1 phosphorylation sites to the Mad1 binding region. The direct interaction was abrogated with Mps1-phosphorylated Megator or in vitro phosphorylated of Megator. Cell-based experiments examining the localization patterns of Mad1 in both MPS1-NLS expressing cells and Megator depleted cells expressing various phospho-site mutants are presented to support the in vitro findings. On this front, the authors provided convincing evidence that that they identified a Megator-Mad1 interaction that is negatively regulated by Mps1.
My major concerns stem from the functional line of enquiry towards analyzing the effects that manipulating this pathway has on the spindle assembly checkpoint and kinetochore localization of Mad1/Mad2. The observation that kinetochore-localization of Mad1 and to a lesser extent C-Mad2 can be restored in Mps1 depleted cells by expressing Megator T24D is both unexpected and hints at a previously unknown regulation mechanism (I wonder if this is conserved?). However, the T4D mutant also yielded results that ran counter to expectations based on prior work on NPC-bound Mad1/Mad2, which warrants further investigation into the production of interphase MCC in the mutants. These concerns as well as others are outlined below.

Major Points:
1) Rodriguez-Bravo et al. (Cell, 2014) observed that expression of Mad1 that could not bind TPR

resulted in more rapid Cyclin B degradation and reduced time to anaphase onset. I was surprised that the T24D mutant, which the authors argue does not localize Mad1 to the NPC, does not result in a shorter time in mitosis compared to the WT Megator cells. Since Rodriguez-Bravo showed that mis-localization of Mad1 from the NPC severely compromised interphase MCC production (as assayed by Cdc20 IPs and blotting for Mad2), it would be worthwhile for interphase MCC assembly to be monitored in the T24D cells.
2) Upon further evaluation of the images and quantification in Figure 3A-C, I wonder if it is possible that there is still residual Mad1 at the NPCs in the T24D cells that is either a consequence of partial depletions of Megator or some remaining Megator binding ability that may account for the discrepancy outlined in point 1. How does the nuclear-envelope associated Mad1 levels in interphase cells compare between Megator depleted cells and Megator depleted + T24D.
3) It is interesting that 2/4 mapped sites (T1295, T1338) are glutamic acid residues in vertebrates. Yet vertebrate TPR still binds Mad1. Could this be through a different interaction region in vertebrate TPR? If so, is this interaction region conserved in flies? If vertebrate and fly TPR use the same region to bind Mad1 then this argues that phosphorylation of T1295 and T1338 is not key to disrupting Mad1 binding. Since the first site may be conserved in vertebrates (there is a conserved Threonine residue one position over) and the fourth site it well-conserved, I think it would be useful to dig into these residues further. For example, would the T1295D/T1338D double mutant still bind Mad1 in vitro and localize it in cells? Alternatively, is a T1259D/T1390D double mutant sufficient to recapitulate what was observed for the 4D mutant? This line of enquiry would better contextualize these findings in terms of evolutionary conservation of the proposed mechanism.
Minor points:
1) The statement markedly reduced Megator hyperphosphorylation on lines 139-140 is a bit over-stated based on the gel, which is not overly convincing.
2) I am curious if the LARIAT approach could be employed to look at mitotic progression some more. If the Mad1 and Mad2 are sequestered away from the kinetochores in the T24A mutant does mitosis proceed more quickly?
3) What do total Mad2 levels (not just C-Mad2) look like in the mutants?
4) The flow of the text is disrupted when the authors discuss the Mad1 and C-Mad2 findings on pp. 6 and 7 as it bounces back and forth between describing results in figures 3 and 4. Summarizing the Mad1 findings together (from Figure 3) and then describing the C-Mad 2 (Figure 4) findings would be smoother.
5) The 2nd MPS1 site is denoted as T1395 throughout the text - I presume the authors mean T1295.
6) I presume the change in anaphase onset from 36 to 39 minutes described on line 203 is not statistically significant. If so then I don't think it's worth stating that it may affect the SAC. (On a related note - see LARIAT point above).
7) Please explain what Jupiter is.
8) What is gEGFP?
9) The font is quite small in a number of figures and it makes it hard to read (Especially figures 1D and 5F).
10) In 1D, it appears the grey line is mis-labeled and should be Mps1 rather than Megator.
11) Replace "endorse" with a more appropriate word on line 241.
12) When discussing the effects of the mutants on lagging chromosomes due to checkpoint independent roles of Mad1 (lines 285-287), I am curious also as to how the authors think checkpoint independent roles of Mad2 in regulating Aurora B (per Kabeche and Compton, Current Biology, 2012) could play a role here.
13) It's strange that the results end (lines 287-297) with references to data reported in a supplemental figure.

Reviewer #2 (Comments to the Authors (Required)):

The manuscript by Osswald et al. is quite comprehensive and the experiments strongly support the main conclusion that Mps1 phosphorylates Megator to release Mad1 so that it can bind to kinetochores. I was impressed that the authors kept pushing to verify their model by looking at all of the molecular players involved, and that they examined aspects of their model not only biochemically, but also looking for all the predicted effects on the cell cycle and chromosome segregation in cultured cells and in vivo. I thus support the publication of this manuscript in the Journal of Cell Biology.

That being said, I must admit to some degree of frustration in reading the manuscript. This was somewhat akin to torture; I felt as if I had to spend a very long time to make headway in getting through it. (a) Several paragraphs go on for more than a page, and it was often difficult to follow the complex logic. (b) Many times I felt the need to ponder what pronouns like "This" were referring to. Usually I could figure out the answer, but the process should not have been so difficult. (c) The introduction does not even mention Mps1; key ideas like its nuclear exclusion during interphase and how it is phosphorylated on its T-loop are sprung by surprise later or never fully explained. These issues mean that the article would only be of interest to the population of people already well versed in the details of the spindle assembly checkpoint. This is not necessarily a small population, but to anyone outside of the field, pretty much the only thing that would be examined is the summary Figure 5F.

Beyond the denseness of the prose, there are a two other major issues I would hope the authors could address.

First, the authors say in their introduction that Drosophila lacks the Mps1-Knl1-Bub1 pathway controlling Mad1 kinetochore localization. This raises the question as to whether the Mps1-Megator pathway they discovered in flies applies to other systems (particularly humans), where the Mps1-Megator pathway may not be needed. The authors do not say whether the phosphorylation sites they discovered in Megator are conserved in other organisms. It would have been very useful if the authors could have done even one experiment pointing to the existence of this pathway in human cultured cells, though I would not demand such an experiment as a precondition for publication.

Second, given the importance of the summary Figure 5F, I was surprised that it does not even suggest the idea that some MCC assembly is taking place at the nuclear pore complex during interphase. This concept is reiterated several times in the text, for example in the last paragraph. The figure just shows the assembly of a Mad1-Mad2 tetramer at the NPC; is this all the authors believe is happening or is there more substantial progress towards MCC assembly?

I would like to see these major points addressed by alterations to the manuscript. However, because I thought the work was so comprehensive, I do not envision the need to conduct more experiments. Several minor issues are listed below; these are more in the way of notes to myself encountered during the effort to plow through the manuscript as opposed to issues that must be formally addressed.

1. Is the Mad1-Mad2 heterotetramer in line 73 the same as Mad1-C-Mad2?
2. Would be helpful in line 77 to state the components of the MCC tetramer because this is not the same as the Mad1-Mad2 heterotetramer.

3. Line 81. Not clear what "sustained SAC signaling" means.
4. Line 86. This heterotetramer is Mad1-C-Mad2?
5. Lines 95-101. If the Mps1-Knl1-Bub1 pathway is missing in Drosophila, what if anything controls the kinetochore localization of Mad1? Are the authors saying that the main control mechanism is Mps1-mediated release of Mad1 from nuclear pores, and no other regulatory step is invovled? And how does the absence of this pathway affect our ability to generalize from the Drosophila case to other systems? (The Discussion should cover this question of generalization.)
6. In the control for Fig. 1, why does it appear that there are multiple microtubule organizing centers, but only two in the Mps1 RNAi sample?
7. The authors should discuss at least in the figure legend why Megator seems to look like it surrounds the nucleus when the nuclear envelope is mostly degraded in prometaphase.
8. Why is tubulin coming into the nucleus faster in the Mps1 RNAi sample?
9. Line 115-117. I am not understanding the sentence saying that the decline in Mad1-EGFP overlaps perfectly with the pattern of Megator-EGFP, given that it was just said
10. In Fig. 2B, Megator appears to be equally phosphorylated in untreated control cells and in control cells treated with colchicine. Yet in Fig, 5F, the authors show MPS1 being excluded from the nucleus and Megator being phosphorylated only beginning in prophase. How can these two statements be reconciled?
11. In Fig. 2B, what is the relationship of the phosphorylation sites in Megator that are shown with respect to known target motifs for Mps1? Also, are these phosphorylation sites well conserved in other species?
12. In Fig. 2I, the differences in the samples are said to be statistically significant, but the data by eyeball don't seem so convincing, as they depend on a few relatively rare outliers. On the other hand, the effects on kinetochore localization are much more persuasive. Perhaps there are many clusters that have just a small amount of colocalized Mad1, so that the pools of Mad1 available to kinetochores are significantly decreased even if this is not so apparent in Fig. 2I?
13. Lines 179-180. It would help readers to understand that Mad1 loss from NPCs in Megator-depleted cells is shown in Fig. 3A but the other results are shown in Fig. 3B and 3C.
14. The results about Mad1 localization at kinetochores in Fig.3D are very nice; again they look better in the pictures than they do in the Fig. 3E graph.
15. Line 190. Would be helpful to know what "this" is that is ameliorated. I figured this out but it took a long time.
16. Lines 222-224. The authors should add a reference to the Figure(s) that show T4D-expressing cells can recruit Mad1 to unattached kinetochores. I presume this is Fig. 3D.E. It is also a bit disconcerting that the results concerning C-Mad2 in these cells were already mentioned previously in Lines 191-193. Nothing wrong with this; but it caused a pause in the reading to try to figure out whether I had read this earlier.
17. Line 228. The antecedent of "these" is unclear. It took me awhile to understand that it is the total levels that are being rescued, not the reduction in the total levels.
18. Line 241. Endorses?
19. Lines 242-244. The authors appear to be arguing that assembly of Mad1-C-Mad2 occurs (exclusively?) at the NPCs and cite a few papers consistent with this view. (By the way, are these papers about the situation in flies or in other organisms; this is not clear?) I don't know this subject intimately, but the is novel to me; is this really a well-accepted view in the field? Does the formation of Mad1-C-Mad2 in solution in vitro? Are there contradictory data indicating that this complex can assemble at kinetochores?
20. Lines 255-256. It is not the inability to localize to kinetochores that is rescued, but the ability to localize to kinetochores.
21. Lines 261-297. I am finding this ridiculously long paragraph almost impossible to follow.
22. Lines 272-275. It was not clear that indistinguishable meant the comparison of Megator and ald

rather than indistinguishable from controls. It would also help to have a consistent usage of mps1-null rather than bringing in the name ald; it takes time to realize that those are the same thing.
23. Line 281. Now we find that Mps1 phosphorylates Mad1. This is not shown in Figure 5.
24. Line 283. Does unperturbed here mean in the absence of colchicine or the absence of mutation/depletion of any components?
25. Lines 283-287. It is not clear what kind of roles the authors envision Mad1 is playing independent of its role in the SAC.
26. Line 301-302. Is it the phosphorylation of Megator by Mps1 that is essential for the kinetochore localization of Mad1-C-Mad2, or is it the abrogation of Megator interaction with Mad1 which is essential? I realize these are in the authors' model related events, but this again shows that it is difficult in this paper to parse out antecedents of prepositions.
27. Lines 305-306 and Figure 5F. Does Mps1 autophosphorylate or is some other kinase responsible.
28. Lines 306-307. These lines again refer to MCC assembly prior to kinetochore maturation yet this is not shown in Figure 5F.

Reviewer #3 (Comments to the Authors (Required)):

Mps1-mediated release of Mad1 from nuclear pores ensures the fidelity of chromosome segregation.

In this paper, the authors show convincingly, in vitro and in vivo, that Mps1 kinase phosphorylates the nucleoporin Tpr (Mtor in Drosophila) at 4 threonines, that these phosphorylations release the binding of Mad1 from Mtor, and that this release is important to the proper functioning of Mad1 during mitosis.

They demonstrate in Drosophila cells (both culture and organism) that the Mps1-mediated release of Mad1 from the nuclear envelope in turn contributes to recruitment of Mad1 on kinetochores during mitosis and so assures proper SAC function. In cells depleted of Mps1, or in cells expressing nonphosphorylatable Mtor mutant proteins (T4A), Mad1 is no longer released from Mtor at mitotic entry and consequently only weakly accumulates on kinetochores. With phosphomimetic (T4D) mutants, Mad1 is no longer associated with Mtor during interphase, but is still recruited to kinetochores. Most impressively, expression of the Mtor T4D mutant protein in mitotically active fly tissues was able to significantly suppress the mitotic phenotypes of two different cell types lacking Mps1 (mutant or RNAi depleted), by allowing Mad1 to accumulate on kinetochores.

This is a good solid study making an important contribution to Drosophia mitosis. With one exception (suppression of intestinal dysplasia Fig S3), the experiments seem well controlled, and the results clear.

Regarding its broader biological significance, it is (as the authors state) the first time Mps1 has been shown to be doing something mitotically important that's not at kinetochore. And it the first description of mechanism for regulated release of Mad1 from the nuclear envelope.

Major Issues

1)It is not clear whether this mechanism will have any relevance to mammalian cells. There should be some discussion of this point.
Only one of the four phosphorylated threonine residues in Megator is conserved in mammals (and

two of the others are acidic residues). Moreover, the domain of mammalian Tpr involved in binding Mad1 has been mapped to a different fragment (1-774, Lee et al 2008, and Rodriguez-Bravo et al 2014) than the one implicated here (1178-1655 in fly Mtor).

The text does not discuss these differences, which is unfortunate. Did the authors try and fail to find any interaction of Mad1 with adjacent N-terminal fragments (in the region 1-1178)? Did they look for phosphorylation-independent interactions? Did they mutate only one of the 4 phospho sites? The conserved one, for example? If such studies were done, it would be useful to know the (presumably negative) results.

2)Mtor /TPR is implicated in chromatin organization and possibly gene expression in both mammalian and Drosophila cells. Can the authors exclude that the phenotypic suppression effects they see are not due to some more systemic physiological alterations caused by the effect of depleting Mtor on gene expression ?This is particularly a problem for the last experiment, the intestinal dysplasia assay.

The "best" control would probably be to identify the region of Mad1 binding to Mtor, mutate that region (while still hopefully maintaining Mad1 functionality), and show that it too could now suppress the Mps1 mitotic phenotypes. The authors will doubtless say (correctly) that this is too big an undertaking for this paper. But at the least, the authors could provide some additional controls. For example, would depleting Mtor reduce dysplasia induced by some method that doesn't involve the SAC (as in the Resende 2018 article) ?

Minor, but still needing attention:

There is almost no discussion of the study by Rodriguez-Bravo et al 2014 who find evidence that Mad1 at NPCs is a source for the generation of some mitotic checkpoint complex (MCC) (the Mad2-Cdc20-BubR1 complex) prior to the assembly of functioning kinetochores at mitotic entry, and that this source of MCC is important for full SAC activity.

In this study, the authors also present evidence that removing Mad1 from the NPCs slightly attenuates the SAC, which they suggest is because less Mad2 binds Mad1 when it is not anchored to Mtor. That is a different explanation than in Rodrigues-Bravo. Moreover, in fly cells where Mps1 activity is inadequate, blocking Mad1 from binding Mps1 actually helps restore the SAC. Could the authors address briefly whether they think their study sheds light (if it does) on the model of Rodriguez-Bravo ? Does NPC-bound Mad1 contribute to the generation of MCC ?

Text and figure errors:
Lines 774-790 and Fig 1 D. The legend does not say what the graphs to the right of the images in D represent. Intensity profiles across the nuclei? That's what they appear to be. (as in Fig S1C ?) . Also, the line colors seem to be improperly defined. Both green and grey lines are called "Megator". Presumably one is Mps1. Which?

Suppl Fig S1D should be S1C.

In the Methods, please provide or give a reference for the RNAi sequences used for the knockdowns in S2 cells.

In general the text was clear and easy to follow. A few English errors should be corrected however:

118: Mad1 "reallocation" should probably be: relocation
129 "albeit" active in the cytoplasm. "Albeit" is a hard word to use correctly. Try to avoid it. "although it is" would be better.

151 and 252: "we resorted to". Better: "we utilised" or "we used" or "we employed". "Resorted to" means we tried all kinds of approaches and none of the others worked.

240-241 "...endorses Mad1-Mad2 to unattached kinetochores..." "Endorses" is the wrong word, but I'm not sure what the authors mean to say. Dissociation helps M1-M2 accumulate on kinetos?

255 "Mad1 inability" should be " Mad1's inability...."

265-6 "rescues" the aneuploidy caused by loss of Mps1. Please be careful with "Rescue". It reduces the aneuploidy frequency. It rescues (or suppresses) the phenotype of Mps1 null (and the phenotype is elevated rates of aneuploidy). But it does not rescue the aneuploidy. 294-5 "suggesting a rescue in the levels of aneuploidy" . Ditto.

277 "albeit" is not right. Try "although"

342 "PCR Reactions" . A bit redundant...

356-358 In the expts described, when were transiently expressing cells used and when were stable lines used? It seems like all the expts were with stable lines. If this is not the case, it should be stated in each experiment.

424-5 "the ROI ... each single kinetochore could fit into IT."

994 "Asterisk denotes.... This is in Fig S2C, but not in S2D.

Response letter

Dear editors,

We would like to thank you for overseeing the review process and the reviewers for their constructive comments on our original work. In this revised version of the manuscript we have carried out additional experiments to address the main points of criticism raised by the reviewers and included additional data that strengthens and expands our previous conclusions.

Point-by-point response to the editors and reviewers:

Dear Dr. Conde,

Thank you for submitting your manuscript entitled "Mps1-mediated release of Mad1 from nuclear pores ensures the fidelity of chromosome segregation". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

The main issue to address for resubmission is the apparent discrepancy between these results as compared to those reported by Rodriguez-Bravo et al. (Cell, 2014). One possibility is the difference at multiple levels between Drosophila and human cells - a point raised by all reviewers. We would recommend checking if the Mtor phosphomutant affects MCC levels as this should provide some clarity into this issue. Regardless, there may still be residual Mad1 at NPCs that is sufficient to generate SAC function in mitosis. In addition to these points, a more thorough discussion of the differences (as pointed out by reviewers) between Mtor in flies and TPR in human cells should be included as well.

We thank the reviewers for the critical and constructive evaluation of the manuscript. We are pleased that all the reviewers recognise the significant interest and potential importance of this work to the field of mitosis. We found their comments and suggestions very useful and accordingly, we performed additional experiments in order to:

1- evaluate the levels of pre-mitotic MCC in S2 cells depleted of endogenous Megator and expressing different EGFP-Megator phosphomutant transgenes. This new data now enables us to better understand and explain why EGFP-Megator^{T4D} cells fail to progress faster through mitosis, as expected in light of previous studies where abolishing the Mad1-Tpr interaction compromised the assembly of pre-mitotic MCC and accelerated mitotic exit (Rodriguez-Bravo et al, 2014). We now show that while depletion of Megator does indeed reduce the levels of pre-mitotic MCC, expression of EGFP-Megator^{T4D} is sufficient to restore MCC assembly to similar levels as the expression of EGFP-Megator^{WT}. This is likely due to a residual pool of Mad1 that persists at NPCs when EGFP-Megator^{T4D} is expressed (point 2) and is therefore still able to catalyse MCC formation to levels that suffice to ensure a normal mitotic timing (point 2).

2- quantitatively compare the levels of Mad1 at NPCs of interphase cells depleted of Megator versus interphase cells depleted of Megator but expressing EGFP-Megator^{T4D}. The new data demonstrate that mimicking constitutive phosphorylation of Megator does exert a negative effect on Mad1 capacity to localize at NPCs but fails however to abolish it completely, as observed upon depletion of Megator. Thus, the pool of Mad1 that remains associated with

NPCs in EGFP-Megator^{T4D}cells is likely sufficient to produce enough pre-mitotic MCC so as not to affect the mitotic timing under unperturbed conditions (point 1).

3- better characterize the Mad1-Megator interaction *in vitro* and contextualize the role of the identified phosphorylations in terms of evolutionary conservation of the proposed mechanism. We performed additional pull-down assays with recombinant MBP-Megator fragments harbouring different phosphomimetic mutations. This enabled us to determine exactly which residues must be phosphorylated to avert binding to Mad1: T1259, T1338 and T1390. Interestingly, T1259 is adjacent to a conserved threonine in vertebrate orthologues, T1390 is well-conserved, and T1338 occurs as glutamic acid in vertebrates Tpr. On the other hand, T1302, which was also mapped as an Mps1-phosphorylatable site, occurs as a glutamine in vertebrates Tpr and its phosphorylation seems to dispensable to prevent the central coiled-coil domain from binding to Mad1.

4- establish the involvement of the proposed mechanism in human cells. We have now conducted experiments with two human cell lines that support Mps1-mediated phosphorylation of Tpr as an evolutionarily conserved mechanism required to release Mad1 from NPCs and enable its appropriate localization on kinetochores. This set of experiments was conducted by Cristina Ferrás, who has now been included as a contributing author in the revised version of the manuscript.

Given that the vast majority of the experiments for this revision were conducted by Sofia Cunha-Silva, we decided that her name should now appear as the first co-first author in the line of authors. All the authors of the manuscript agree with this alteration.

All text modifications introduced in the revised version of the manuscript are highlighted in blue.

Reviewer #1 (Comments to the Authors (Required)):

Osswald and Cunha-Silva present a series of compelling studies examining the regulation of the Mad1-Megator interface by Mps1 kinase in this generally well-written manuscript. The authors identified a region of Megator that directly binds Mad1 in vitro and mapped four Mps1 phosphorylation sites to the Mad1 binding region. The direct interaction was abrogated with Mps1-phosphorylated Megator or in vitro phosphorylated of Megator. Cell-based experiments examining the localization patterns of Mad1 in both MPS1-NLS expressing cells and Megator depleted cells expressing various phospho-site mutants are presented to support the in vitro findings. On this front, the authors provided convincing evidence that that they identified a Megator-Mad1 interaction that is negatively regulated by Mps1.
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the production of interphase MCC in the mutants. These concerns as well as others are outlined below.

Major Points:
1) Rodriguez-Bravo et al. (Cell, 2014) observed that expression of Mad1 that could not bind TPR resulted in more rapid Cyclin B degradation and reduced time to anaphase onset. I was surprised that the T24D mutant, which the authors argue does not localize Mad1 to the NPC, does not result in a shorter time in mitosis compared to the WT Megator cells. Since Rodriguez-Bravo showed that mis-localization of Mad1 from the NPC severely compromised interphase MCC production (as assayed by Cdc20 IPs and blotting for Mad2), it would be worthwhile for interphase MCC assembly to be monitored in the T24D cells.

As suggested by the reviewer, we assessed the levels of interphase MCC in parental cells depleted of endogenous Megator and in cells transfected with RNAi-resistant EGFP-Megator^{WT}, EGFP-Megator^{T4D} and EGFP-Megator^{T4A} transgenes. This new data is presented in Figure 4H in the revised version of the manuscript. In agreement with previous work from Rodriguez-Bravo et al (2014), depletion of Megator reduced the levels of Cdc20 that co-immunoprecipitated with BubR1 in lysates of asynchronous cultured cells (> 97% interphase cells). Accordingly, depletion of Megator from parental cells resulted in a mild, yet statistically significant ($P = 0.0102$), acceleration of mitotic progression under otherwise unperturbed conditions (24 min in control cells versus 20 min in Megator RNAi cells). These results confirm the so-called "mitotic timer" to be compromised in the absence of Megator/Tpr as previously proposed by Rodriguez-Bravo et al (2014). Notably, expression of EGFP-Megator^{T4D} rescued the assembly of interphase MCC to the same level as observed in interphase cells expressing EGFP-Megator^{WT}. Concurrently, EGFP-Megator^{T4D} cells were able to undergo mitosis with a similar mitotic timing as EGFP-Megator^{WT} cells in the absence of spindle poisons. It is important to note that although Megator^{T4D} binds significantly less to Mad1 *in vitro*, the interaction is not completely abolished. Accordingly, cells expressing EGFP-Megator^{T4D} retain a residual pool of Mad1 at NPCs, which might be sufficient to support assembly of pre-mitotic MCC (please see Reviewer #1 point 2).

2) Upon further evaluation of the images and quantification in Figure 3A-C, I wonder if it is possible that there is still residual Mad1 at the NPCs in the T24D cells that is either a consequence of partial depletions of Megator or some remaining Megator binding ability that may account for the discrepancy outlined in point 1. How does the nuclear-envelope associated Mad1 levels in interphase cells compare between Megator depleted cells and Megator depleted + T24D.

We thank the reviewer for pointing out this important control, as, together with the previous point, it allowed us to clarify the apparent discrepancy with Rodriguez-Bravo et al (2014). To quantitatively compare the levels of NPC-associated Mad1 in cells expressing EGFP-Megator^{T4D} with the ones of Megator-depleted parental cells we have now quantified the immunofluorescence signal of Mad1 relative to Nup107, a core nucleoporin whose levels at NPCs are not affected by the absence of Megator. The results are presented in Figure S1E,F of the revised manuscript and show that cells expressing EGFP-Megator^{T4D} still retain a residual pool of Mad1 at NPCs. This markedly contrasts with parental cells depleted of Megator, where Mad1 localization at the nuclear envelope is virtually abolished. Considering the levels of interphase MCC found in these cells (please see Reviewer #1 point 1 and Figure 4H of the revised manuscript), we agree with the reviewer that phosphomimetic Megator retains some ability to bind Mad1, which may account for the apparent discrepancy with

Rodriguez-Bravo et al (2014). This point is now discussed in the revised version of the manuscript.

3) It is interesting that 2/4 mapped sites (T1295, T1338) are glutamic acid residues in vertebrates. Yet vertebrate TPR still binds Mad1. Could this be through a different interaction region in vertebrate TPR? If so, is this interaction region conserved in flies? If vertebrate and fly TPR use the same region to bind Mad1 then this argues that phosphorylation of T1295 and T1338 is not key to disrupting Mad1 binding. Since the first site may be conserved in vertebrates (there is a conserved Threonine residue one position over) and the fourth site it well-conserved, I think it would be useful to dig into these residues further. For example, would the T1295D/T1338D double mutant still bind Mad1 in vitro and localize it in cells? Alternatively, is a T1259D/T1390D double mutant sufficient to recapitulate what was observed for the 4D mutant? This line of enquiry would better contextualize these findings in terms of evolutionary conservation of the proposed mechanism.

We thank the reviewer for this important suggestion. We delved further into this matter and have now established which phosphorylations are critical to inhibit the interaction between Mad1 and the central coiled-coil domain of Megator *in vitro*. It is important to mention here, that in the original version of the manuscript, one of the identified Mps1-phosphorylation sites (**T1302**) was incorrectly attributed to T1295 in the alignment of Megator/Tpr orthologues of Figure 2D. T1295 was never detected in our MS analysis as a residue phosphorylated by Mps1. We apologize for this mistake and now present in the revised version of the manuscript the residue T1302 correctly annotated in Figure 2D.
Since T1259 and T1390 appear to be conserved in vertebrate Tpr (as mentioned by the reviewer, T1259 is adjacent to a conserved threonine and T1390 is well-conserved) we followed the reviewer's suggestion and assessed *in vitro* whether phosphorylation of these two residues was sufficient to recapitulate the defective Mad1-binding capacity of Megator^{T4D}. However, contrasting with MBP-Megator$^{1187\text{-}1655/T4D}$, the double phosphomimetic for T1259 and T1390 (MBP-Megator$^{1187\text{-}1655/T2D}$) is still efficiently pulled-down by 6xHis-Mad1$^{1\text{-}493}$. Interestingly, further converting T1338 to aspartate (MBP-Megator$^{1187\text{-}1655/T3D}$), compromised the interaction with Mad1 to a similar extent as MBP-Megator$^{1187\text{-}1655/T4D}$. These results indicate that the cumulative phosphorylation of the potentially conserved T1259 and T1390 residues is not sufficient to dissociate Mad1 from *Drosophila* Megator, which further requires a third phosphorylation on T1338. Since in vertebrates Tpr this residue is naturally replaced by a negatively charged amino acid, it is possible that in vertebrates the phosphorylation of the two conserved threonines is sufficient to promote the release of MAD1 from Tpr. The results also suggest that phosphorylation of T1302 is functionally irrelevant for the proposed mechanism. In line with this, T1302 corresponds to a polar uncharged glutamine in vertebrate Tpr. This new set of results is now presented in Figure S1C,D and discussed in the revised version of the manuscript.

Minor points:
1) The statement markedly reduced Megator hyperphosphorylation on lines 139-140 is a bit over-stated based on the gel, which is not overly convincing.

We agree with the reviewer and removed "markedly".

2) I am curious if the LARIAT approach could be employed to look at mitotic progression some more. If the Mad1 and Mad2 are sequestered away from the kinetochores in the T24A mutant does mitosis proceed more quickly?

Following the Reviewer's suggestion, we monitored the mitotic progression of S2 cells after light-induced clustering of EGFP-Megator$^{1187\text{-}1655/WT}$ and EGFP-Megator$^{1187\text{-}1655/T4A}$. In the absence of LARIAT clustering, S2 cells expressing EGFP-Megator$^{1187\text{-}1655/WT}$ and EGFP-Megator$^{1187\text{-}1655/T4A}$ fragments progress from NEB to anaphase onset with similar timings. This result is in line with the absence of striking differences in the mitotic timing of S2 cells expressing either the full length versions of EGFP-Megator^{WT} or EGFP-Megator^{T4A} (Figure 4G). Interestingly, when the formation of clusters was induced, EGFP-Megator$^{1187\text{-}1655/T4A}$ cells seem to exit slightly faster from mitosis than EGFP-Megator$^{1187\text{-}1655/WT}$ cells (23 min versus 26 min, median time). Unfortunately, we only managed to score a very limited number of cells by live-imaging, thus precluding a robust statistical analysis of this experiment. It is tempting to suggest that clustering somehow increases EGFP-Megator$^{1187\text{-}1655/T4A}$ capacity to sequester Mad1 away from kinetochores in a way that decreases the efficiency of SAC signaling. Nevertheless, because of the marginal effect observed and the reduced sample size, we do not feel comfortable in including this result in the manuscript. We opted to present it in this letter as **Figure R1**.

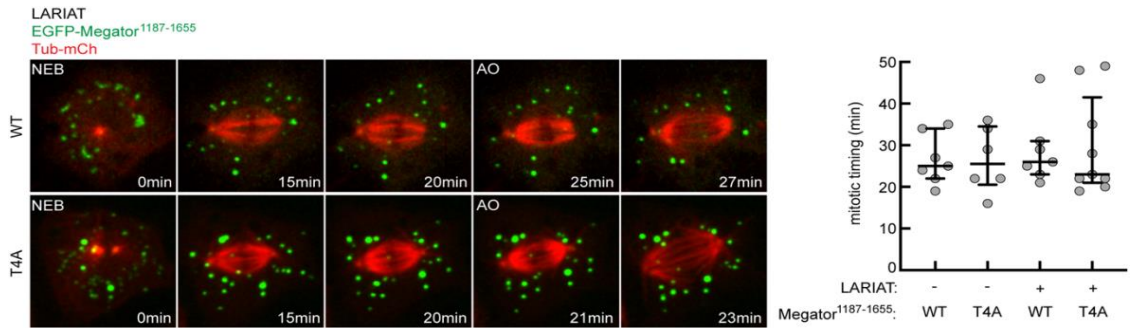

Figure R1. Mitotic progression of *Drosophila* S2 cells expressing Tubulin-mCherry, EGFP-Megator$^{1187\text{-}1655}$ transgenes and LARIAT modules (CIBN-MP and CRY2-VHH) was monitored through time-lapse microscopy. Mitotic timing was defined as the time cells took from nuclear envelope breakdown (NEB) to anaphase onset (AO). Graph shows median mitotic timing with interquartile range in the presence and absence of LARIAT-mediated EGFP-Megator$^{1187\text{-}1655}$ clustering. Clustering was triggered through exposure to blue light for 30 minutes before imaging and was continuously stimulated during imaging by the 488nm laser used to image EGFP-Megator transgenes.

3) What do total Mad2 levels (not just C-Mad2) look like in the mutants?

To address this, we assessed by immunofluorescence the levels of Mad2 at unattached kinetochores of parental cells depleted of endogenous Megator and in cells transfected with RNAi-resistant EGFP-Megator^{WT}, EGFP-Megator^{T4D} and EGFP-Megator^{T4A} transgenes. We used a Mad2 antibody (Rb1223) that recognizes both the O-Mad2 and C-Mad2 conformers (Orr et al., 2007 - PMID:17182852) and CID staining was used as a kinetochore reference.

The results recapitulate the findings obtained for C-Mad2: depletion of Megator causes a significant reduction in amount of Mad2 present at unattached kinetochores of parental S2 cells. A similar reduction is observed in cells expressing either EGFP-Megator^{T4A} or EGFP-Megator^{T4D} transgenes when compared to EGFP-Megator^{WT} cells. Given the limitations in the number of supplementary figures and the confirmatory character of this result, we opted to present it in this letter as **Figure R2.**

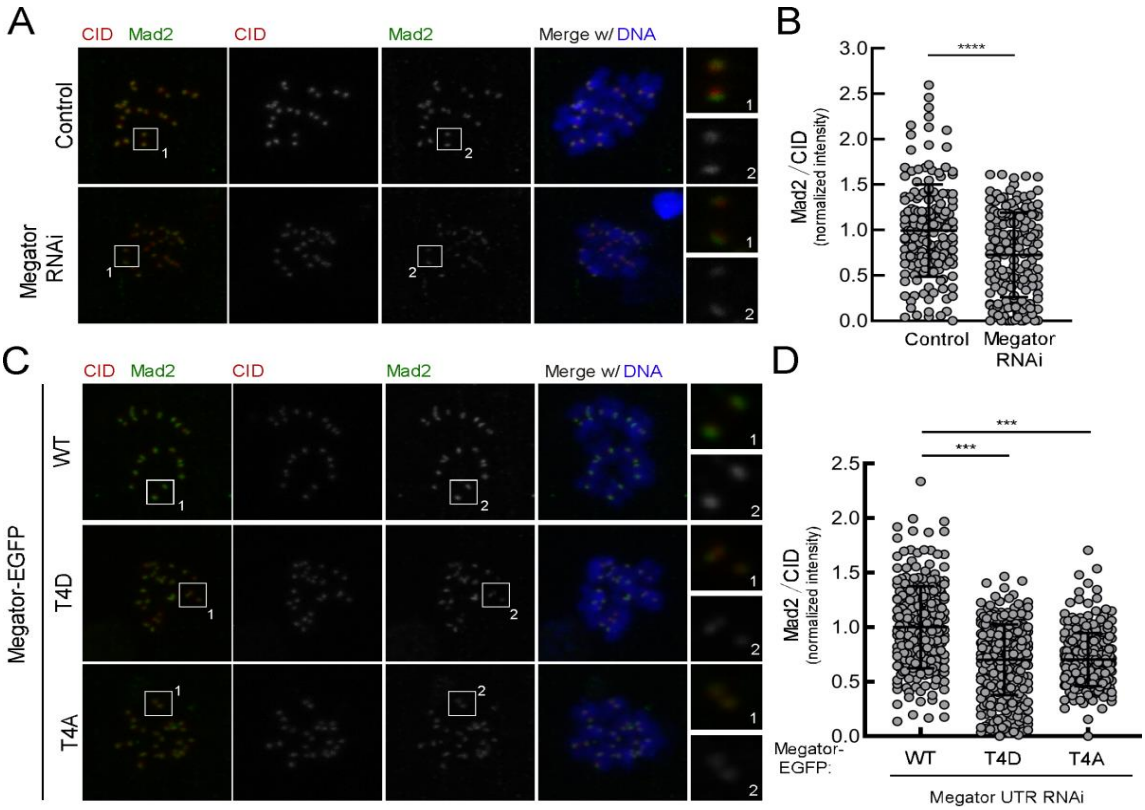

Figure R2. Constitutively impaired Mad1-Megator interaction reduces Mad2 levels at kinetochores. (A,B) Representative immunofluorescence images (A) and corresponding quantifications (B) of Mad2 levels at unattached kinetochores of control or Megator-depleted S2 cells. Mad2 fluorescence intensities were determined relative to CID signal (N ≥ 167 kinetochores for each condition). The values obtained for control cells were set to 1. (C,D) Representative immunofluorescence images (C) and corresponding quantifications (D) of Mad2 at unattached kinetochores of S2 cells depleted of endogenous Megator and expressing the indicated Megator-EGFP transgenes. Mad2 fluorescence intensities were determined relative to CID signal (N ≥115 kinetochores for each condition). The values obtained for Megator^{WT}-EGFP were set to 1. The insets display magnifications of the outlined regions. Cells were incubated MG123 (20µM) for 3h and with colchicine (30µM) for 2h. Expression of Megator-EGFP transgenes in was induced for 24 hours prior processing for immunofluorescence analysis or live cell imaging. Data information: data are presented as mean ±SD. Asterisks indicate that differences between mean ranks are statistically

significant, ***p<0.001, ****p<0.0001, (Student's t-test in (B) and Kruskal-Wallis, Dunn´s multiple comparison test in (D)).

4) The flow of the text is disrupted when the authors discuss the Mad1 and C-Mad2 findings on pp. 6 and 7 as it bounces back and forth between describing results in figures 3 and 4. Summarizing the Mad1 findings together (from Figure 3) and then describing the C-Mad 2 (Figure 4) findings would be smoother.

Following the reviewer's suggestion, we have now described the Mad1 and C-Mad2 findings separately.

5) The 2nd MPS1 site is denoted as T1395 throughout the text - I presume the authors mean T1295.

The second Mps1 phosphorylation site is actually T1302 (please see Reviewer #1 major point 3). We thank the reviewer for noticing this error, which has now been corrected in the revised version of the manuscript.

6) I presume the change in anaphase onset from 36 to 39 minutes described on line 203 is not statistically significant. If so then I don't think it's worth stating that it may affect the SAC. (On a related note - see LARIAT point above).

As suggested, we removed this statement from the revised version of the manuscript.

7) Please explain what Jupiter is.

The *Drosophila* protein Jupiter is a microtubule-associated protein, MAP (Karpova et al., 2006 - PMID:16518797). The Jupiter:GFP fusion protein reproduces microtubule behavior, so it is often used in transgenic fly lines to visualize the mitotic spindle as an alternative to Tubulin transgenes (Karpova et al., 2006). We have now specified Jupiter as a MAP in the legend of Figure 5C in the revised manuscript.

8) What is gEGFP?

In fly genetics, gEGFP is often used to denote a EGFP-tagged transgene whose expression is controlled by the *cis*-regulatory region of the corresponding gene. In our study, we opted to maintain the original nomenclature of the *gEGFP-Mps1* transgenes described by Althoff et al (2012 - PMCID:PMC3374747).

9) The font is quite small in a number of figures and it makes it hard to read (Especially figures 1D and 5F).

We have modified the font in Figures 1D and 5F in the revised version of the manuscript.

10) In 1D, it appears the grey line is mis-labeled and should be Mps1 rather than Megator.

We thank the reviewer for pointing this out. We have corrected this in the revised manuscript.

11) Replace "endorse" with a more appropriate word on line 241.

We replaced “endorse” with “enables”

12) When discussing the effects of the mutants on lagging chromosomes due to checkpoint independent roles of Mad1 (lines 285-287), I am curious also as to how the authors think checkpoint independent roles of Mad2 in regulating Aurora B (per Kabeche and Compton, Current Biology, 2012) could play a role here.

Kabeche and Compton (2012, PMCID: PMC3326208) have shown that Mad2 stabilizes kinetochore-microtubule attachments during mitosis independently of its SAC function and through a yet unclarified Aurora B inhibitory mechanism. Although we don’t exclude the involvement of this pathway in control neuroblasts, it is unlikely to contribute for the observed rescue in the accuracy of chromosome segregation in *mps1-null* mutants co-depleted of Megator since Mad2 kinetochore localization remains compromised in the absence of Megator.

13) It's strange that the results end (lines 287-297) with references to data reported in a supplemental figure.

We agree. Although we would prefer otherwise, to preserve the flow of the reading we had to maintain this arrangement. Nevertheless, the conclusions and description of the model are supported with a reference to a main figure (Figure 5F).

Reviewer #2 (Comments to the Authors (Required)):

The manuscript by Osswald et al. is quite comprehensive and the experiments strongly support the main conclusion that Mps1 phosphorylates Megator to release Mad1 so that it can bind to kinetochores. I was impressed that the authors kept pushing to verify their model by looking at all of the molecular players involved, and that they examined aspects of their model not only biochemically, but also looking for all the predicted effects on the cell cycle and chromosome segregation in cultured cells and in vivo. I thus support the publication of this manuscript in the Journal of Cell Biology.

That being said, I must admit to some degree of frustration in reading the manuscript. This was somewhat akin to torture; I felt as if I had to spend a very long time to make headway in getting through it. (a) Several paragraphs go on for more than a page, and it was often difficult to follow the complex logic. (b) Many times I felt the need to ponder what pronouns like "This" were referring to. Usually I could figure out the answer, but the process should not have been so difficult. (c) The introduction does not even mention Mps1; key ideas like its nuclear exclusion during interphase and how it is phosphorylated on its T-loop are sprung by surprise later or never fully explained. These issues mean that the article would only be of interest to the population of people already well versed in the details of the spindle assembly checkpoint. This is not necessarily a small population, but to anyone outside of the field, pretty much the only thing that would be examined is the summary Figure 5F.

We thank the reviewer for pointing out these concerns with the text. We modified it in an attempt to address points (a) and (b). However, in what concerns point (c), the requirement to keep the text character count < 20,000 severely limits our ability to address current knowledge on Mps1 regulation, including its nuclear exclusion and T-loop autophosphorylation. Since we are submitting the manuscript for consideration as a report,

we felt that referring to these concepts when demanded by the narrative, as opposed to a detailed description in the introduction, would still allow for a succinct, yet sufficient understanding of the key ideas. However, if the reviewer or the editors feel that a more extensive and detailed description of some concepts should be provided, we will gladly work on the text to accomplish it.

Beyond the denseness of the prose, there are a two other major issues I would hope the authors could address.

First, the authors say in their introduction that Drosophila lacks the Mps1-Knl1-Bub1 pathway controlling Mad1 kinetochore localization. This raises the question as to whether the Mps1-Megator pathway they discovered in flies applies to other systems (particularly humans), where the Mps1-Megator pathway may not be needed. The authors do not say whether the phosphorylation sites they discovered in Megator are conserved in other organisms. It would have been very useful if the authors could have done even one experiment pointing to the existence of this pathway in human cultured cells, though I would not demand such an experiment as a precondition for publication.

This point converges with the major point (3) of Reviewer #1. As can be seen from the Clustal Omega (EMBL-EBI) local sequence alignment presented in Figure 2D, two of the four Mps1-phosphorylation sites identified in Megator are potentially conserved in vertebrate orthologues: T1259 is adjacent to a conserved threonine and T1390 is well-conserved. Moreover, T1338 is replaced by a negatively charged amino acid in vertebrates Tpr - glutamic acid. As mentioned in the response to Reviewer #1, phosphorylation of these three residues is sufficient and required to compromise *in vitro* the interaction between Mad1 and the central coiled-coil domain of Megator (Figure S1C,D). Hence, we envision that Mps1-mediated phosphorylation of Tpr might also take place in human cells to release Mad1 from NPCs. To test whether this mechanism also contributes to ensure robust kinetochore recruitment of Mad1 in human cells, we inactivated Mps1 with reversine in RPE and HeLa cells and examined their capacity to accumulate Mad1 at unattached kinetochores upon siRNA-mediated depletion of Tpr (Figure S3). As expected, inhibition of Mps1 severely compromised kinetochore recruitment of Mad1. However, knocking-down Tpr partially restored Mad1 capacity to localize at kinetochores in the presence of reversine (Figure S3). These results are now presented and discussed in the revised version of the manuscript and concur to suggest that a similar kinetochore-extrinsic mechanism orchestrated by Mps1 also operates in human cells to release Mad1 from NPCs and facilitate its kinetochore recruitment.

Second, given the importance of the summary Figure 5F, I was surprised that it does not even suggest the idea that some MCC assembly is taking place at the nuclear pore complex during interphase. This concept is reiterated several times in the text, for example in the last paragraph. The figure just shows the assembly of a Mad1-Mad2 tetramer at the NPC; is this all the authors believe is happening or is there more substantial progress towards MCC assembly?

The model in Figure 5F aims to summarize the main findings of this study. Although it is well established that MCC assembly also takes place during interphase, exactly how the C-Mad2 that is activated at NPCs by Mad1-C-Mad2 intersects with Cdc20 and with BubR1-Bub3 remains elusive. Hence, we opted to avoid depicting unnecessary speculative mechanisms in the model as this could possibly divert the reader from the main conclusions and novelty of our work. Nevertheless, we have now extended our model to include a more

complete picture of the processes underlying: (i) MCC assembly at NPCs during interphase, (ii) Mps1-mediated release of Mad1-C-Mad2 from NPCs in prophase and (iii) kinetochore-mediated catalysis of MCC formation during prometaphase. This extended version of the model is now presented in Figure 5F of the revised manuscript.

I would like to see these major points addressed by alterations to the manuscript. However, because I thought the work was so comprehensive, I do not envision the need to conduct more experiments. Several minor issues are listed below; these are more in the way of notes to myself encountered during the effort to plow through the manuscript as opposed to issues that must be formally addressed.

1. Is the Mad1-Mad2 heterotetramer in line 73 the same as Mad1-C-Mad2?

It is and it has now been corrected accordingly.

2. Would be helpful in line 77 to state the components of the MCC tetramer because this is not the same as the Mad1-Mad2 heterotetramer.

As suggested, we have now described in the text the components of the MCC.

3. Line 81. Not clear what "sustained SAC signaling" means.

We replaced “sustained” by “robust”

4. Line 86. This heterotetramer is Mad1-C-Mad2?

Yes it is and it has now been specified in the revised text.

5. Lines 95-101. If the Mps1-Knl1-Bub1 pathway is missing in Drosophila, what if anything controls the kinetochore localization of Mad1? Are the authors saying that the main control mechanism is Mps1-mediated release of Mad1 from nuclear pores, and no other regulatory step is invovled? And how does the absence of this pathway affect our ability to generalize from the Drosophila case to other systems? (The Discussion should cover this question of generalization.)

In addition to Mps1-mediated release of Mad1 from NPCs, other regulatory steps are likely involved in controlling Mad1 recruitment to unattached kinetochores. Our results show that Mad1 has to be released from nuclear pores, so it is “free” to go to kinetochores. We would argue that conceptually this is not incompatible with additional regulatory events, that might for instance, actively promote the ability of kinetochores to bind Mad1 previously released from nuclear pores. Thus, we do not think the absence of the Mps1-Knl1-Bub1 pathway precluded us from finding a conserved mechanism. In fact, we have now included and discussed in the revised version of the manuscript new data supporting that Mps1-mediated release of Mad1 from NPCs is evolutionarily conserved in vertebrates.
Moreover, although the Mps1-Knl1-Bub1 pathway is dispensable in *Drosophila* cells to recruit Mad1 to kinetochores, several lines of evidence support the notion that additional mechanisms must exist in *Drosophila*: (i) expression of EGFP-Megator^{T4A} does not completely abolish Mad1 kinetochore recruitment, (ii) expression of EGFP-Megator^{T4D} does not completely restore kinetochore recruitment of Mad1 in cells depleted of Mps1 and (iii) depletion of Megator fails to rescue to 100% the recruitment of Mad1 to unattached

kinetochores in the absence of Mps1 activity. Mad1 kinetochore recruitment in flies is known to depend on kinetochore-associated RZZ complex. Whether the RZZ-dependent and the kinetochore-extrinsic pathways intercept and/or additional mechanisms exist is unclear.

6. In the control for Fig. 1, why does it appear that there are multiple microtubule organizing centers, but only two in the Mps1 RNAi sample?

Aberrant number of centrosomes is quite typical in *Drosophila* S2 cells. It has been previously shown that more than 50% of *Drosophila* S2 cells contain multiple centrosomes (Kwon et al., 2008; Gergely and Basto, 2008).

7. The authors should discuss at least in the figure legend why Megator seems to look like it surrounds the nucleus when the nuclear envelope is mostly degraded in prometaphase.

Drosophila cells undergo a semi-closed mitosis. As suggested by the reviewer, this is now mentioned in the legend of Figure 1.

8. Why is tubulin coming into the nucleus faster in the Mps1 RNAi sample?

We do not have an explanation for this observation.

9. Line 115-117. I am not understanding the sentence saying that the decline in Mad1-EGFP overlaps perfectly with the pattern of Megator-EGFP, given that it was just said

This sentence has now been modified in the revised version of the manuscript.

10. In Fig. 2B, Megator appears to be equally phosphorylated in untreated control cells and in control cells treated with colchicine. Yet in Fig, 5F, the authors show MPS1 being excluded from the nucleus and Megator being phosphorylated only beginning in prophase. How can these two statements be reconciled?

Several kinases might phosphorylate Megator/Tpr before mitotic entry. For instance, Tpr has been shown to be phosphorylated by the MAP kinase ERK2 (Eblen et al., 2003- PMID:12594221) and by protein kinase A (Rajanala et al., 2014- PMID: 24938596).

11. In Fig. 2B, what is the relationship of the phosphorylation sites in Megator that are shown with respect to known target motifs for Mps1? Also, are these phosphorylation sites well conserved in other species?

To our knowledge, a well-defined consensus signature for Mps1 phosphorylation sites remains to be established. However, one common feature that comes out from comparing most of Mps1substrates identified so far is the occurrence of a negatively charged residue at position -2 or at position -3 (Dou et al., 2011- PMID:21533207; Hennrich et al., 2013; PMID: 23510141; Maciejowski et al., 2017-PMID: 28441529). In that respect, it is interesting to note that T1259 has a phosphorylatable serine at -2; T1302 has a negatively charged glutamic acid at -2; T1338 has a phosphorylatable serine at -3 and T1390 has a negatively charged glutamic acid at -2.
The evolutionary conservation of these sites has been addressed above in response to major point (3) of Reviewer #1 and to major point (1) of Reviewer #2. Two of the four Mps1-phosphorylation sites are potentially conserved in vertebrate orthologues: T1259 is adjacent

to a conserved threonine and T1390 is well-conserved. T1302 and T1338 are respectively replaced by a conserved glutamine and glutamic acid in vertebrates.

12. In Fig. 2I, the differences in the samples are said to be statistically significant, but the data by eyeball don't seem so convincing, as they depend on a few relatively rare outliers. On the other hand, the effects on kinetochore localization are much more persuasive. Perhaps there are many clusters that have just a small amount of colocalized Mad1, so that the pools of Mad1 available to kinetochores are significantly decreased even if this is not so apparent in Fig. 2I?

The reviewer is correct.

13. Lines 179-180. It would help readers to understand that Mad1 loss from NPCs in Megator-depleted cells is shown in Fig. 3A but the other results are shown in Fig. 3B and 3C.

This has been modified in the revised manuscript.

14. The results about Mad1 localization at kinetochores in Fig.3D are very nice; again they look better in the pictures than they do in the Fig. 3E graph.

We are pleased that the Reviewer appreciates these results.

15. Line 190. Would be helpful to know what "this" is that is ameliorated. I figured this out but it took a long time.

This is now modified in the revised version of the manuscript.

16. Lines 222-224. The authors should add a reference to the Figure(s) that show T4D-expressing cells can recruit Mad1 to unattached kinetochores. I presume this is Fig. 3D.E. It is also a bit disconcerting that the results concerning C-Mad2 in these cells were already mentioned previously in Lines 191-193. Nothing wrong with this; but it caused a pause in the reading to try to figure out whether I had read this earlier.

As suggested, references to Figures have been added. The results concerning C-Mad2 are now described in the text after the results concerning Mad1.

17. Line 228. The antecedent of "these" is unclear. It took me awhile to understand that it is the total levels that are being rescued, not the reduction in the total levels.

We have modified the text accordingly to avoid misinterpretation.

18. Line 241. Endorses?

We replaced “endorses” by “enables”

19. Lines 242-244. The authors appear to be arguing that assembly of Mad1-C-Mad2 occurs (exclusively?) at the NPCs and cite a few papers consistent with this view. (By the way, are these papers about the situation in flies or in other organisms; this is not clear?) I don't know this subject intimately, but the is novel to me; is this really a well-accepted view in the field?

Does the formation of Mad1-C-Mad2 in solution in vitro? Are there contradictory data indicating that this complex can assemble at kinetochores?

The cited papers report the assembly of a stable Mad1-C-Mad2 complex in Xenopus egg extracts and in human cultured cells. However, is not clear where and how Mad1-C-Mad2 assembly occurs in cells. We do not exclude that Mad1-C-Mad2 might also assemble at kinetochores during mitosis. However, Mad1-C-Mad2 complexes are already present at NPCs and in the nucleoplasm before mitotic entry. Our results suggest that Megator/Tpr is not only required to localize Mad1-C-Mad2 at NPCs, but might also operate as a scaffold to facilitate Mad1-C-Mad2 interaction before mitosis: (i) depletion of Megator reduces C-Mad2 levels at kinetochores (~ 50%), (ii) replacing endogenous Megator by a Mad1-binding defective phosphomutant reduces C-Mad2 levels at kinetochores (~ 50%), (iii) decreased kinetochore localization of C-Mad2 in (i) and (ii) is not caused by altered Mad1 or Mad2 proteostasis and (iv) decreased kinetochore localization of C-Mad2 in (i) and (ii) occurs despite normal levels of Mad1 at kinetochores.

20. Lines 255-256. It is not the inability to localize to kinetochores that is rescued, but the ability to localize to kinetochores.

We thank the reviewer for pointing this out, and have now modified it accordingly.

21. Lines 261-297. I am finding this ridiculously long paragraph almost impossible to follow.

We have shortened this paragraph in the revised version of the manuscript.

22. Lines 272-275. It was not clear that indistinguishable meant the comparison of Megator and ald rather than indistinguishable from controls. It would also help to have a consistent usage of mps1-null rather than bringing in the name ald; it takes time to realize that those are the same thing.

We have modified both issues accordingly.

23. Line 281. Now we find that Mps1 phosphorylates Mad1. This is not shown in Figure 5.

Mps1-mediated phosphorylation of Mad1 on T716 promotes the binding of Cdc20 N-terminal tail to Mad1 C-terminal domain (Ji et al., 2017 - PMID: 28072388; Faesen et al., 2017- PMID:28102834; Ji et al., 2018 - PMID:29162720). This event represents a critical step in SAC activation as it accelerates the formation of C-Mad2-Cdc20 and consequently MCC assembly (Faesen et al., 2017; Ji et al., 2017). Although obviously important, this mechanism does not represent a critical central point in our narrative and that is why its absent from Figure 5F. Phosphorylation of Mad1 by Mps1 is mentioned in the text in order to explain why cells depleted of Megator and Mps1 are still SAC-defective despite able to recruit Mad1.

24. Line 283. Does unperturbed here mean in the absence of colchicine or the absence of mutation/depletion of any components?

Unperturbed was used to denote the absence of spindle poisons. We have now clarified this in the respective Figure legends.

25. Lines 283-287. It is not clear what kind of roles the authors envision Mad1 is playing independent of its role in the SAC.

The role that Mad1 plays in kinetochore-microtubule attachments is still rather elusive. It has been shown in flies that mad1-null mutant neuroblasts accumulate merotelic attachments and consequently display high frequencies of lagging chromatids (Emre et al., 2011- PMID: 21511728). These observations suggest the involvement of Mad1 in the correction/prevention of erroneous attachments. Although the underlying mechanism remains unclear, Emre et al (2011) have shown that it does not require the presence of Mad2, but it does depend in some unknown way on key residues in the Mad2-binding domain of Mad1. Interestingly, Mad1 directly interacts and recruits Cut7 (Eg5 homologue) and CENP-E to misaligned kinetochores in fission yeast and human cells, respectively (Akera et al., 2015 - PMID:26258632). Although neither of these microtubule-binding motors have kinetochore-microtubule correction activity, it is tempting to speculate that if spindle bipolarity is compromised (defective Cut7 recruitment) or if chromosome congression is impaired (defective CENP-E recruitment), the occurrence of merotely is expected to increase. Because these hypotheses have not been directly tested, the SAC-independent role that Mad1 plays to ensure the accuracy of kinetochore-microtubule attachments remains to be established and therefore was not deeply covered in the manuscript.

26. Line 301-302. Is it the phosphorylation of Megator by Mps1 that is essential for the kinetochore localization of Mad1-C-Mad2, or is it the abrogation of Megator interaction with Mad1 which is essential? I realize these are in the authors' model related events, but this again shows that it is difficult in this paper to parse out antecedents of prepositions.

We modified the sentence to "Phosphorylation of Megator by Mps1 abrogates Megator interaction with Mad1 and this is essential for kinetochore localization of Mad1-C-Mad2…"

27. Lines 305-306 and Figure 5F. Does Mps1 autophosphorylate or is some other kinase responsible.

We modified the text in the revised manuscript.

28. Lines 306-307. These lines again refer to MCC assembly prior to kinetochore maturation yet this is not shown in Figure 5F.

This issue has been addressed in response to the second major point of Reviewer #2.

Reviewer #3 (Comments to the Authors (Required)):

Mps1-mediated release of Mad1 from nuclear pores ensures the fidelity of chromosome segregation.

In this paper, the authors show convincingly, in vitro and in vivo, that Mps1 kinase phosphorylates the nucleoporin Tpr (Mtor in Drosophila) at 4 threonines, that these phosphorylations release the binding of Mad1 from Mtor, and that this release is important to the proper functioning of Mad1 during mitosis.

They demonstrate in Drosophila cells (both culture and organism) that the Mps1-mediated release of Mad1 from the nuclear envelope in turn contributes to recruitment of Mad1 on kinetochores during mitosis and so assures proper SAC function. In cells depleted of Mps1, or in cells expressing nonphosphorylatable Mtor mutant proteins (T4A), Mad1 is no longer released from Mtor at mitotic entry and consequently only weakly accumulates on kinetochores. With phosphomimetic (T4D) mutants, Mad1 is no longer associated with Mtor during interphase, but is still recruited to kinetochores. Most impressively, expression of the Mtor T4D mutant protein in mitotically active fly tissues was able to significantly suppress the mitotic phenotypes of two different cell types lacking Mps1 (mutant or RNAi depleted), by allowing Mad1 to accumulate on kinetochores.

This is a good solid study making an important contribution to Drosophia mitosis. With one exception (suppression of intestinal dysplasia Fig S3), the experiments seem well controlled, and the results clear.

Regarding its broader biological significance, it is (as the authors state) the first time Mps1 has been shown to be doing something mitotically important that's not at kinetochore. And it the first description of mechanism for regulated release of Mad1 from the nuclear envelope.

Major Issues

1)It is not clear whether this mechanism will have any relevance to mammalian cells. There should be some discussion of this point. Only one of the four phosphorylated threonine residues in Megator is conserved in mammals (and two of the others are acidic residues). Moreover, the domain of mammalian Tpr involved in binding Mad1 has been mapped to a different fragment (1-774, Lee et al 2008, and Rodriguez-Bravo et al 2014) than the one implicated here (1178-1655 in fly Mtor). The text does not discuss these differences, which is unfortunate. Did the authors try and fail to find any interaction of Mad1 with adjacent N-terminal fragments (in the region 1-1178)? Did they look for phosphorylation-independent interactions? Did they mutate only one of the 4 phospho sites? The conserved one, for example? If such studies were done, it would be useful to know the (presumably negative) results.

The relevance of the proposed mechanism to mammalian cells is now discussed in the revised version of the manuscript. As can be seen from the Clustal Omega (EMBL-EBI) local sequence alignment presented in Figure 2D, two of the four Mps1-phosphorylation sites identified in Megator are potentially conserved in vertebrate orthologues: T1259 is adjacent to a conserved threonine and T1390 is well-conserved. This raised the question whether phosphorylation of these two sites was sufficient to regulate Mad1 binding. However, pull-down assays revealed that introducing double phosphomimetic mutations in these sites is not sufficient to efficiently disrupt the interaction with Mad1 (Figure S1C,D). This is however accomplished when T1338 is also converted to aspartic acid (Figure S1C,D). Since T1338 is naturally replaced by a glutamic acid in vertebrates Tpr, it is plausible that a similar mechanism operates in mammals to disrupt the Mad1-Megator interaction. To directly test whether this mechanism is relevant in human cells, we assessed the capacity of Tpr-depleted RPE and Tpr-depleted HeLa cells to recruit Mad1 to kinetochores in the presence of the Mps1 inhibitor reversine. As observed in *Drosophila* S2 cells and neuroblasts, depletion of Tpr partially restored Mad1 kinetochore localization in human cells lacking Mps1 activity (Figure S3). These results, which are now presented and discussed in the revised version of the manuscript, support that a similar kinetochore-extrinsic mechanism controlled by Mps1

operates in human cells to ensure the release of MAD1 from Tpr at NPCs and enable Mad1 recruitment to kinetochores.

Finally, we would like to mention that a similar direct, although weaker, interaction between Mad1 and Tpr$^{774\text{-}1700}$ (corresponding to Megator$^{1187\text{-}1655}$) was also reported by Lee et al (2008) (please refer to pull-down assays on Figure 1F from Lee et al., 2008).
As suggested by the reviewer, we examined *in vitro* the ability of Mad1 N-terminus to interact with different regions of Megator. The results are presented in Figure S1B of the revised manuscript. As reported by Lee et al (2008) and Rodriguez-Bravo et al (2014) for mammalian Tpr, we found that Mad1 is also able to bind to the more N-terminal coiled-coil domains of *Drosophila* Megator (equivalent to Tpr$^{1\text{-}774}$). This binding interface does not seem to be subjected to phospho-regulation by Mps1 (we could never detect phosphorylation of these fragments by Mps1 in the *in vitro* kinase assays). Interestingly however, it was recently shown in human cells that Cyclin B1 binding to Mad1 also contributes to the proper release of Mad1 from NPCs (Jackman et al., 2019- https://doi.org/10.1101/701474) and for its timely recruitment to kinetochores (Alfonso-Pérez et al., 2019- PMID: 30674583 ; Jackman et al., 2019; Allan et al., 2019 https://doi.org/10.1101/726224). Therefore, we speculate that Cyclin B1-CDK1 and Mps1 may cooperate to release Mad1 from NPCs by disrupting two different but adjacent binding interfaces. In line with a synergistic mechanism, it was recently shown that a mutant Mad1 that cannot bind Cyclin B1 remains associated with Tpr in early mitosis and this association was significantly increased by inhibition of Mps1 with low doses of reversine. This point has now been discussed in the revised version of the manuscript.

2)Mtor /TPR is implicated in chromatin organization and possibly gene expression in both mammalian and Drosophila cells. Can the authors exclude that the phenotypic suppression effects they see are not due to some more systemic physiological alterations caused by the effect of depleting Mtor on gene expression ?This is particularly a problem for the last experiment, the intestinal dysplasia assay.

The "best" control would probably be to identify the region of Mad1 binding to Mtor, mutate that region (while still hopefully maintaining Mad1 functionality), and show that it too could now suppress the Mps1 mitotic phenotypes. The authors will doubtless say (correctly) that this is too big an undertaking for this paper. But at the least, the authors could provide some additional controls. For example, would depleting Mtor reduce dysplasia induced by some method that doesn't involve the SAC (as in the Resende 2018 article) ?

The reviewer raises an important point. As suggested, we addressed the possibility that the suppression of intestinal dysplasia observed when Megator was depleted could be caused by a different role of Megator, not specific to SAC function. We started by assessing the phenotype that the depletion of Megator by itself causes on intestinal stem cells (ISC). Expression of UAS-MegatorRNAi results by itself in considerable loss of ISCs (**Figure R3**). This observation suggests that the suppression of tissue dysplasia driven by depletion of Megator in an Mps1-depleted background may simply be caused by ISCs death and not necessarily due to a rescue in the fidelity of chromosome segregation and genome stability. To further test this possibility, we followed the reviewer's suggestion and examined whether depletion of Megator rescues intestinal dysplasia induced through a pathway that is not expected to compromise the SAC. For this, we used flies carrying a UAS-Raf construct for overexpression. The Raf oncogene encodes a serine-threonine protein kinase that activates the MEK/ERK pathway to regulate cell proliferation, differentiation and survival (Lu et al., 1994- PMID: 8013459). Overexpression of Raf in ISCs induces severe intestinal dysplasia in

a loss-of-SAC independent manner (Ma et al., 2016- PMID: 26845534). However, when UAS-MegatorRNAi was co-expressed with UAS-Raf we observed a dramatic reduction in tissue dysplasia (**Figure R3**). Collectively, this set of results supports the possibility advanced by the reviewer, that the suppression of intestinal dysplasia reported in the initial submission likely results from other alterations caused by loss of Megator and affecting ISCs maintenance. Although this does not exclude that depletion of Megator may also improve chromosome segregation in dividing ISCs, it means that evaluating intestinal dysplasia is not a good readout to test this hypothesis. Accordingly, we have removed from the revised version of the manuscript the data concerning intestinal dysplasia.
However, it should be noted that the results obtained with the adult intestines do not affect in any way the interpretation of the results from the neuroblasts experiments. In this system, the fidelity of chromosome segregation and genome stability were assessed directly by scoring chromosome mis-segregation events in dividing cells, as well as by looking at the karyotype. Hence, the original conclusion that depletion of Megator rescues the mitotic fidelity and genome stability in mps1-null mutants *in vivo* remains unaltered.

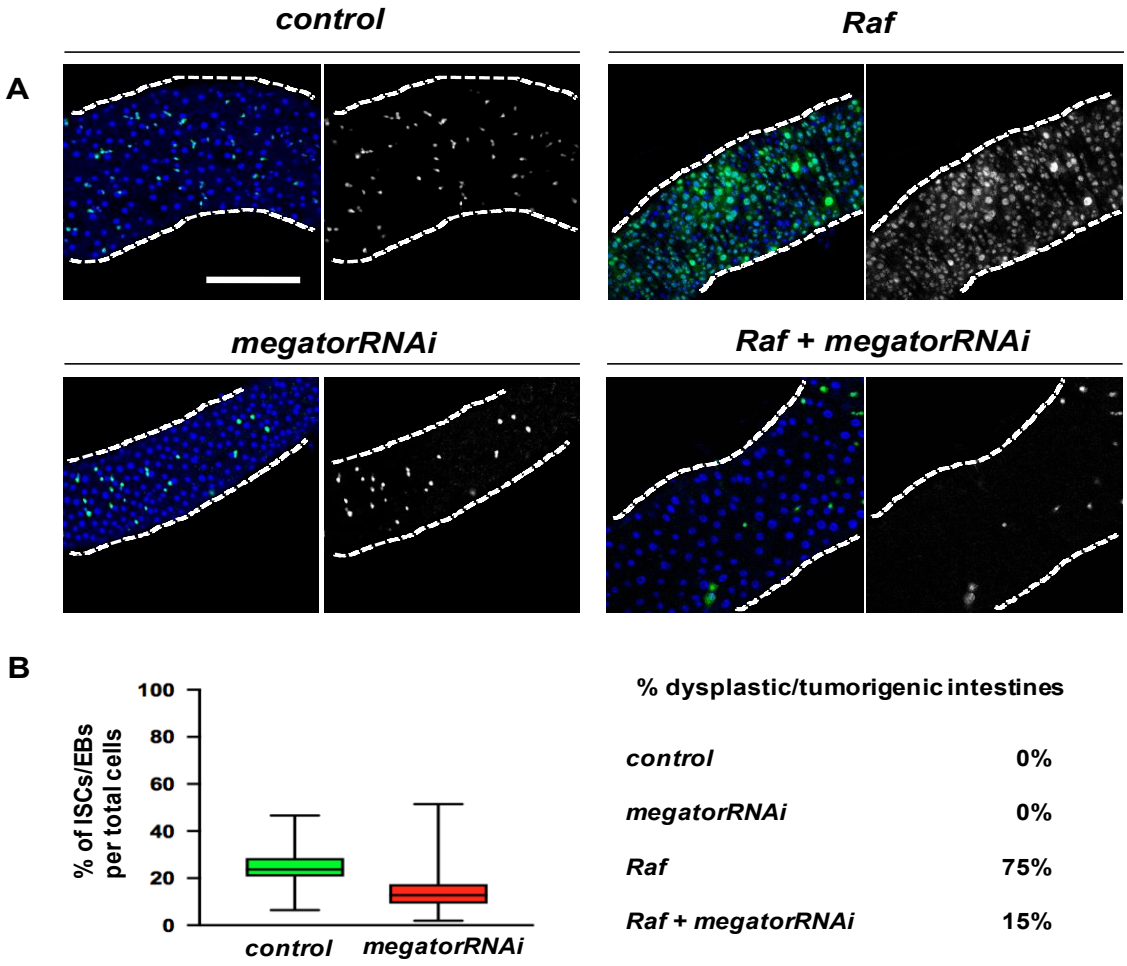

Figure R3: Depletion of Megator causes loss of ISCs and rescues tissue dysplasia in a SAC-independent manner. A) Representative images of posterior midguts of indicated genotypes; scale bar=100µm, all images are in the same magnification. B) Quantification of phenotypes

indicated for genotypes in A); N>20 (intestines) for ISC/EB quantification(left), N>12 (intestines) for evaluation of dysplasia/tumorigenic phenotype (right, table).

Minor, but still needing attention:

There is almost no discussion of the study by Rodriguez-Bravo et al 2014 who find evidence that Mad1 at NPCs is a source for the generation of some mitotic checkpoint complex (MCC) (the Mad2-Cdc20-BubR1 complex) prior to the assembly of functioning kinetochores at mitotic entry, and that this source of MCC is important for full SAC activity.

In this study, the authors also present evidence that removing Mad1 from the NPCs slightly attenuates the SAC, which they suggest is because less Mad2 binds Mad1 when it is not anchored to Mtor. That is a different explanation than in Rodrigues-Bravo. Moreover, in fly cells where Mps1 activity is inadequate, blocking Mad1 from binding Mps1 actually helps restore the SAC. Could the authors address briefly whether they think their study sheds light (if it does) on the model of Rodriguez-Bravo ? Does NPC-bound Mad1 contribute to the generation of MCC ?

This point converges with previous ones raised by Reviewer #1 and Reviewer #2. We have now included a more thorough discussion of the study by Rodriguez-Bravo et al (2014). Our study does not exclude the assembly of pre-mitotic MCC and its importance to define the "minimum" mitotic timing. In fact, we have now confirmed in the revised version of the manuscript that assembly of interphase MCC also occurs in Drosophila S2 cells and in a Megator-dependent manner (Figure 4H of the revised manuscript). However, the new data now included in the manuscript led us to conclude that impaired assembly of pre-mitotic MCC cannot account for the weakened SAC function observed in cells expressing EGFP-Megator^{T4D}. These cells exhibit limited binding of Mad1 to Megator, but it is still sufficient to enable a residual pool of Mad1 to localize at NPCs (Figure S1E,F of the revised manuscript) and catalyze the formation of pre-mitotic MCC to levels similar to those detected in controls (Figure 4H of the revised manuscript). Nevertheless, these cells fail to arrest in mitosis when treated with colchicine as efficiently as cells expressing EGFP-Megator^{WT}. Notably, although EGFP-Megator^{T4D} cells are proficient in recruiting Mad1 to unattached kinetochores, the levels of kinetochore-associated C-Mad2 (and total Mad2) are reduced by 50%, thus correlating with the observed SAC attenuation. Therefore, in addition to its role in docking Mad1-C-Mad2 at NPCs to promote pre-mitotic MCC assembly, we propose that Megator may also serve as a scaffold to facilitate the interaction between Mad1 and Mad2 and accumulate sufficient levels of Mad1-C-Mad2 complexes before mitotic entry.

Text and figure errors:
Lines 774-790 and Fig 1 D. The legend does not say what the graphs to the right of the images in D represent. Intensity profiles across the nuclei? That's what they appear to be. (as in Fig S1C ?) . Also, the line colors seem to be improperly defined. Both green and grey lines are called "Megator". Presumably one is Mps1. Which?

We thank the reviewer for pointing these out. We have now added the description of the intensity profiles across the nuclei to the legend of Figure 1D.

Suppl Fig S1D should be S1C.

We thank the reviewer for noticing this. We have modified it accordingly.

In the Methods, please provide or give a reference for the RNAi sequences used for the knockdowns in S2 cells.

The sequence of primers used in the synthesis of RNAi is now included in the Material and Methods section.

In general the text was clear and easy to follow. A few English errors should be corrected however:

118: Mad1 "reallocation" should probably be: relocation

We have modified accordingly.

129 "albeit" active in the cytoplasm. "Albeit" is a hard word to use correctly. Try to avoid it. "although it is" would be better.

As suggested, we replaced “albeit” by “although it is”.

151 and 252: "we resorted to". Better: "we utilised" or "we used" or "we employed". "Resorted to" means we tried all kinds of approaches and none of the others worked.

As suggested, we replaced “resorted to” by “we used”

240-241 "...endorses Mad1-Mad2 to unattached kinetochores..." "Endorses" is the wrong word, but I'm not sure what the authors mean to say. Dissociation helps M1-M2 accumulate on kinetos?

We modified this accordingly.

255 "Mad1 inability" should be " Mad1's inability...."

We thank the reviewer for pointing this out. The text is now modified in the revised version of the manuscript.

265-6 "rescues" the aneuploidy caused by loss of Mps1. Please be careful with "Rescue". It reduces the aneuploidy frequency. It rescues (or suppresses) the phenotype of Mps1 null (and the phenotype is elevated rates of aneuploidy). But it does not rescue the aneuploidy. 294-5 "suggesting a rescue in the levels of aneuploidy" . Ditto.

We thank the reviewer for pointing this out. We have modified it accordingly.

277 "albeit" is not right. Try "although"

As suggested, we replaced “albeit” by “although”.

342 "PCR Reactions" . A bit redundant...

We have removed “reactions”

356-358 In the expts described, when were transiently expressing cells used and when were stable lines used? It seems like all the expts were with stable lines. If this is not the case, it should be stated in each experiment.

All cell lines used in this work were established as stable cell lines.

424-5 "the ROI ... each single kinetochore could fit into IT."

This has been corrected

994 "Asterisk denotes.... This is in Fig S2C, but not in S2D.

The asterisk denotes bands resulting from unspecific anti-GFP blotting (Figure S2C). In the western-blot depicted in Figure S2D we did not use the anti-GFP antibody.

November 12, 2019

RE: JCB Manuscript #201906039R

Dr. Carlos Conde
IBMC, Instituto de Biologia Molecular e Celular, Universidade do Porto
Rua Alfredo Allen, 208
Porto 4200-135
Portugal

Dear Dr. Conde:

Thank you for submitting your revised manuscript entitled "Mps1-mediated release of Mad1 from nuclear pores ensures the fidelity of chromosome segregation". We would be happy to publish your paper in JCB pending final text changes and revisions necessary to meet our formatting guidelines (see details below).

- Provide main and supplementary text as separate, editable .doc or .docx files
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- Add scale bars to insets Fig 2G, 3A, 3C, 4C, 4E, 5A, S1A, S1E, S2A, S2E, S3A and S3B; and Fig 5C,
- Add MW markers to Fig 2C
- Add a paragraph after the Materials and Methods section briefly summarizing the online supplementary materials

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Sincerely,

Timothy Yen, Ph.D.
Monitoring Editor

Marie Anne O'Donnell, Ph.D.
Scientific Editor

Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

The revised manuscript from Cunha-Silva and Osswald has adequately addressed my major concerns with the addition of new data on interphase MCC assembly and re-analysis of NPC localization of Mad1 using Nup107 to ratio the Mad1 signal in Megator depleted cells expressing the T4D mutant. These data now explain the discrepancy between the mitotic timing differences in Megator depleted (shorter) and Megator-depleted cells expressing the T4D mutant. The data suggest that there are additional Mad1 recruitment sites in Megator/TPR that are likely not regulated by Mps1-mediated phosphorylation that are sufficient to drive MCC assembly from NPCs. I appreciate the authors' efforts in addressing this source of confusion. The discussion (and some supporting data) on the evolutionary conservation of this phospho-regulatory mechanism is also a nice addition to the resubmission. I have some minor textual issues that should be addressed.
1) Regarding the sentence in the abstract: "We find that Mps1 phosphorylates Megator/Tpr to abolish its interaction with Mad1 in vitro and in Drosophila cells." I think the data more strongly supports the in vitro claim, but the fact that the T4D mutant still recruits Mad1 AND there is even still some nuclear Mad1 evident in the cells over-expressing the MPS1-NLS (Figure 1D, E) does not

support the use of the word "abolish" when referring to cell-based data. Please edit this sentence accordingly to better match the data in the paper.
2) Similar concern for the sentence on p. 7 lines 203-205. Should read something like (recommendation in CAPS) "..results support that (FULL or COMPLETE) kinetochore recruitment of Mad1 requires its dissociation from Megator.." Could probably lose "of the latter" in this sentence as well. For reference, the phrasing of the sentence beginning "Thus, we reason..." on p. 7, lines 218-221 represent a more accurate description of the data.

Reviewer #3 (Comments to the Authors (Required)):

In my opinion authors have done a thorough, commendable job replying to all the major critiques, certainly my own. Extending the messages of paper to mammalian cells significantly improves the study's value (new Fig S3, replacing an ambiguous experiment measuring suppression of intestinal dysplasia in fly gut). The writing is much lighter too, even though it covers some topics more comprehensively than did the original submission (Rodriguez-Bravo for example).

This is a fine study.

Missing a word line 225: Despite BEING fully competent in recruiting Mad1...