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Drosophila Polo regulates the Spindle Assembly Checkpoint through Mps1-dependent BubR1 phosphorylation

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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.)

1st Editorial Decision 20 November 2012

Thank you for submitting your manuscript on Polo regulation of Mps1 in the Drosophila spindle checkpoint for consideration by The EMBO Journal. We have now received the feedback from three expert referees, whose comments are copied below. As you will see, these referees consider your results important in principle, but they also raise various major points that would need to be addressed before publication. A number of these concerns regard the presentation and interpretation of the work, and should be addressable by substantial and careful rewriting efforts; while other issues (especially but not exclusively in the report from referee 3) will also require additional (control) experimentation.

Should you be able to address the various points raised to the referees' satisfaction, then we should be able to consider a revised version of this manuscript further for publication. Please do keep in mind that it is our policy to allow only a single round of major revision, and that it is therefore important to diligently and comprehensively respond to all points at this stage. When preparing your letter of response to the referees' comments, please also remember that this will form part of the Review Process File, and will therefore be available online to the community for more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html).

We generally allow three months as standard revision time, and it is our policy that competing manuscripts published during this period will have no negative impact on our final assessment of your revised study. However, we request that you contact the editor as soon as possible upon

publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

Conde et al. address the functional relationship between the Polo and Mps1 kinases in the spindle assembly checkpoint (SAC) in Drosophila. They provide an extensive set of data from analyses mainly with cultured S2 cells and some with larval neuroblasts. They use established methodology. They demonstrate that

1. Polo depletion or addition of BI2536 result in a mitotic arrest that does not depend on the SAC proteins Mad2 and BubR1. Cdc20-IPs from the arrested cells have lower amounts of associated Mad2 and BubR1.

2. Polo is shown to be a positive regulator of Mps1 kinetochore (kt) localization to which also Aurora B (AurB) and Ndc80 contribute.

3. T182 phosphorylation on Polo, which is known to be brought about by AurB and to stimulate Polo activity, is shown to be correlated with inter-sister kt tension, and a corresponding phospho-mimik mutant Polo version promotes Mps1 kt localization even when AurB is inhibited.

5. Polo appears to have its effects on the intensity of 3F3/2 signals which are normally correlated with inter-sister kt tension indirectly via Mps1.

4. BubR1 phosphorylation but not kt localization depend on Mps1.

5. Mps1 appears to promote kt recruitment of Cdc20 by BubR1 and thereby promote formation of the complexes that inhibit the APC/C.

These findings are clearly novel in the Drosophila system. Most of these issues have previously been analyzed in human cells where the detailed functional characterization of the SAC is clearly more advanced than in Drosophila and where a far greater number of studies have been published, presumably because of the link to human cancer. Several findings by Conde et al. further emphasize that some mechanistic details of SAC function in humans and Drosophila are clearly different. The comparison between these two animal systems therefore points to the adaptive plasticity of the SAC. These differences should presumably be considered as a warning that SAC plasticity might also be displayed by different human cell types which not all might behave like HeLa cells. The eventual publication of this technically solid study will therefore be of interest for SAC research.

Apart from some minor problems, the following two appear to be major to me:
1. Conde et al are to be applauded for their comprehensive approach where they look at many SAC components. There is one obvious experiment that seems to be crucial for understanding the role of Polo and AurB for Mps1 kt localization. As also observed in experiments with human cells, depletion or inhibition of these two kinases appears to have a partial effect on Mps1 kt localization. Human AurB for example is only required for a rapid association of Mps1 with the kt. Eventually, Mps1 levels are quite normal. Similarly, the manipulation of Polo and AurB preformed by Conde et al. do not abolish Mps1 kt localization completely. As also indicated in their model, these two

kinases might act redundantly to some extent. This should be addressed by double depletion/inhibition experiments. These experiments will be important in clarifying how well we understand Mps1 kt localization.

2. The second major problem concerns the writing of the manuscript. In an attempt to integrate the vast SAC literature from vertebrates, Conde et al. mention various literature reports in a highly redundant fashion (often three times: in the introduction, in the result and the discussion section). As a result, the manuscript is very hard to read and not as concise as it should be. For example, Conde et al start the result section by stating that data concerning the role of Plk1 for SAC and 3F3/2 would be conflicting, and they continue "The conflicting data from vertebrate model systems prompted us to examine the role of Drosophila Polo in SAC signalling." I do not quite understand this logic. A conflict with data from vertebrate model systems cannot be resolved by looking at yet another system. Science does not work like democracy. (for another example see minor point 3) I am convinced that this manuscript would profit tremendously from extensive re-writing, aiming at a relatively short introduction (where the players are briefly introduced without unfolding details of disagreement) followed by a concise description of the experiments and the corresponding results (without debating their relation to the work in other systems) that are then discussed in relation to what has been found in other systems. With other words, the recommendation is to move most of the references concerning other systems, discrepancies and open questions to the discussion. Moreover, because this work clearly concerns Drosophila where the mechanistic details of SAC function are clearly not identical to all other eukaryotes, Conde et al should make this clear already in the title. In general, they should make it clearer what they think is of general validity and what might be Drosophila-specific. Some minor points to consider in this regard: Vleugel et al. 2012 have recently also pointed out interesting differences concerning the kinase domain in Drosophila BubR1. What do Conde et al. mean when they write about Cenp-E in Drosophila? Cenp-meta or Cenp-ana?

Minor points (order reflects appearance in text and not importance)
1. p.7: "Having established that Polo is required for the MCC formation, we ...":
"is required for the" should be replaced by "promotes".

2. p.7: "... resulted in a significant reduction of the checkpoint kinase Mps1 at unattached kinetochores."
It is not clear to me how Conde et al. were able to decide in their experiments, whether a kinetochore is unattached or not. In a recent publication, the same lab has reported that in polo[9] mutants, chromosomes attach in a syntelic fashion. Therefore, just selecting cells where chromosomes are not neatly aligned in a metaphase plate is unlikely to assure that the kts are not attached.

3. p. 11: "Recent data in yeast and human cells showed that Mps1 phosphorylates KNL1/Spc105/Spc7 in its MELT repeats to promote direct binding of Bub3 to KNL1 thus allowing recruitment of Bub1 and BubR1 (London et al, 2012; Shepperd et al, 2012; Yamagishi et al, 2012)." and p22: "However, Drosophila Spc105/Knl-1 possesses species-specific MELT-like motifs that lack the phosphorylatable threonine but instead contain an excess of negative charges that possibly bypasses phosphodependency of recruitment controlled by Mps1 (Schittenhelm et al, 2009; Vleugel et al, 2012).
Schittenhelm et al. 2009 have demonstrated that BubR1 is still present at kts in Spc105 null mutants and also in internal deletion mutants lacking the "MELT"-repeats. Thus the suggestion that acidity of the Drosophila MELT-repeats seems to be an inappropriate speculation for explaining the discrepancy between Drosophila and humans. (As Schittenhelm et al. 2009 have made it already fairly clear that the role of Spc105 in BubR1 kt localization in Drosophila seems different from that in yeast and human cells, it makes little sense to mention the work in these alternative systems on p.11 before presenting the experiments concerning the role of Mps1 for BubR1 kt localization in Drosophila.)

4. It is quite striking that Mps1 IPs contain Ndc80 and BubR1 IPs do not (Fig. 4E). Moreover, Cdc20 IPs do not contain phosphorylated BubR1 (Fig. 7C). These results are not mentioned in the text. They are not really in line with the suggested model (Mps1 phosphorylates BubR1 at the kt which then can recruit Cdc20 efficiently). These observations need to be discussed. Similarly, why is phosphorylated BubR1 not apparent in Fig. 1D?

5. p.18: "This markedly contrasts with cells that exclusively expressed endogenous Mps1, in which establishment of tension upon chromosome biorientation, precluded Mps1 localization at kinetochores and consequently resulted in a significant reduction of 3F3/2 and Cdc20 kinetochore levels (Figure 7A, B)."
As far as I understand, Fig 7A,B do not include data from cells that express exclusively endogenous Mps1. This data seems to be "not shown".

6. p.23: Conde et al discuss the role of Mps1 and Polo in generation of the 3F3/2 epitope. It would be of interest to reference Dou et al. 2011 which have suggested that these two kinases might have largely overlapping substrate specificity.

7. p.23: "Importantly, constitutive kinetochores localization of Mps1 ...": It would be better to state here (as also later in the discussion) that Mps1 overexpression was used to enforce persistent kt localization. Mps1 fusions with kt localization signals to enforce constitutive localization in various systems have been reported but this is not what Conde et al. have done.

8. supplemental information: p.11 description of kt signal intensity quantification is not clear. Individual kts are said to have been selected manually, but later in the sentence a ROI is mentioned that includes all kts. Please clarify.

9. typographical errors:
Fission -> fission (p. 16)
occurs -> occur (p. 19)
hyperphosphorytated (p. 24)
neuroblats (p. 36)

in Suppl. information:
describe -> described (p. 11)
ressuspended, Mercaptoetanol, Glicerol (p. 12)
p. 13: does the "(x)" after anti-Fizzy/Cdc20 and anti-Cdc27 need to be there?

Referee #2 (Remarks to the Author):

The manuscript entitled "Polo regulates the SAC through MPS1-dependent BubR1 phosphorylation" presents very well documented new findings exploring the interplay between Aurora B, MPS1, the Ndc80/Nuf2 complex, and Polo. However, in my opinion, some of the data are over-interpreted, which precludes from ending with a clear picture of this interplay. Nevertheless, my concerns are more about the writing than the data.
1- As a major breakthrough, Conde et al. show that in the absence of Polo protein or kinase activity, cells are delayed in metaphase independently of Mad2 or BubR1. As the authors propose, this could reflect an essential role for Polo in regulating the APC/C. Of course, it would have added huge value if they could have demonstrated it directly. I assume that the authors failed to measure APC/C activity onto Cdc27 immunoprecipitates (for which they have antibodies, as shown in Fig. 6D), but if this is not the case, they should definitely try this experiment.
The authors then ask whether Polo could also have a role in controlling the checkpoint. They show that the formation of the APC/C inhibitor (MCC: Cdc20-BubR1-Mad2 complex) is impaired in Polo defective cells. This finding argues that the arrest observed upon Polo deficiency might indeed be independent of the checkpoint, since the formation of MCC is partially impaired in this condition. Showing that Mad2 is not recruited to the kinetochores in the absence/inhibition of Polo would definitely strengthen the point that the arrest is independent from the SAC (without this easy experiment, one can still wonder if Polo could have a role in silencing an even weakened checkpoint, for instance). Indeed, it is unclear to me why they need to overactivate the checkpoint with colchicine to demonstrate that Polo deficiency arrests the cells in metaphase independently of the checkpoint. If Polo is required to activate the APC/C, why do we need to activate the checkpoint to uncover it?
2- The authors clearly show that upon Polo inhibition/reduction, MPS1 kinetochore recruitment and overall phosphorylation levels are reduced, which could explain why less MCC is produced upon Polo inhibition/reduction. However, the concept that MPS1 could auto-activate upon dimerization and autophosphorylation at the kinetochore was proposed (Kang et al., 2007; Jelluma et al., 2008),

but not demonstrated, and is not the sole mechanism. It has also been shown that MPS1 is activated via CDK1-dependent phosphorylation, independently of the kinetochore (Morin et al., Curr. Biol., 2012). The authors should not ignore that we still know little about MPS1 mode of activation. What we do know, is that MPS1 is heavily phosphorylated, but showing a reduction in the intensity of lower-migrating bands, (i) does not mean lack of phosphorylation at the kinetochore, and (ii) does not necessarily mean lack of activity. Indeed, some phosphorylation(s) could be inhibitory, and hyperphosphorylation (directly by Polo for instance?) often leads to a smear in the migration pattern which can be misinterpreted as a diminution of phosphorylation (faster migrating forms will diminish the intensity of each individual band). In other words, to claim that they are monitoring MPS1 activity by looking at its phosphorylation, the authors should use a phospho-specific antibody known to detect an activatory phosphorylation. Otherwise, they can just claim that MPS1 phosphorylation is affected in absence of Polo activity, which is already interesting.
3- Using an Aurora B phospho-mimicking Polo mutant (Polo-T182D), the authors elegantly demonstrate that Polo is required upstream of MPS1, favoring its recruitment at the kinetochore, independently of Aurora B. They further show that, on the contrary, MPS1 recruitment at kinetochores is dependent on Ndc80/Nuf2. To strengthen this point, they used high-resolution imaging to show that Polo-T182 phosphorylation and MPS1 are concomitantly lost upon increase of the interkinetochore distance (and concomitant escape from Aurora-B dependent phosphorylation). Strikingly, Polo-T182 constitutive phosphorylation is sufficient to recruit MPS1 onto these distant, correctly attached, late prometaphase kinetochores. This really argues for a direct role of Polo in recruiting MPS1 at the kinetochore in drosophila.
4- Another well documented finding is the role for MPS1 in controlling BubR1 & 3F3/2 hyperphosphorylation at the kinetochore (but not BubR1 recruitment to the kinetochore). In sharp contrast with previous studies, the fact that the 3F3/2 staining disappears upon Nuf2/Ndc80 depletion (which prevents MPS1, but not Polo recruitment at the kinetochore) argues for MPS1 being a more direct kinase than Polo to generate the 3F3/2 epitope.
Then, they show that in addition to members of the Aurora B Polo -> Ndc80/Nuf2 -> MPS1 -> P-BubR1 pathway, Bub3 is also required to recruit Cdc20 at the kinetochore. Altogether, this suggests that phospho-BubR1 is an important player of Cdc20 recruitment at the kinetochore. This is further supported by the results showing that upon artificial maintenance of MPS1 -> P-BubR1/3F3 at kinetochores in late prometaphase (when kinetochores are attached and stretched), -> Cdc20 is also maintained at kinetochores.
The authors finally show that EGFP-Mps1 is required to stabilize the formation of the Cdc20-BubR1 complex in the cytoplasm, but that this does not sustain the checkpoint as efficiently as in the presence of kinetochore-dependent catalysis, confirming previous findings obtained in human cell cultures (Maciejowski et al., 2010).

Various issues:
- I have a concern regarding the use of the Polo9 mutant, described in Donaldson et al., J. Cell Biol., 2001. In this publication (that the current manuscript forgets to cite), they were claiming that Polo deficiency was causing a metaphase arrest with centromere separation. This discrepancy of phenotype should be discussed in the current manuscript. I am not aware of any follow-up on this "metaphase arrest with centromere separation" phenotype, but I would be glad to know.
- The sentence p.9: "(...) and suggest that Polo may control MPS1 recruitment to kinetochores indirectly by promoting the correct localization and activation of Aurora B" is misleading since the paper later demonstrates that this is not the case. The sentence could stop before the "and" without missing anything.
- The legends embedded in the immunofluorescence images are very difficult to read on screen, and impossible to read once printed (although the figures are still at full scale). These labels should be enlarged and bolded, and/or printed on white layer, or indicated above the figure.
- p.10; p.14, ...: it should be mentioned whether they are referring to inter- or intra-kinetochore tension.

Referee #3 (Remarks to the Author):

Review of Conde et al, EMBOJ-2012-83630

Polo regulates the spindle assembly checkpoint through Mps1-dependent BubR1 phosphorylation

Conde and colleagues investigated the role of Polo kinase in the spindle assembly checkpoint in Drosophila cells. They demonstrated that the active Polo kinase is essential for kinetochore recruitment of Mps1. They subsequently showed that Mps1 kinetochore localization is required for BubR1 hyperphosphorylation and formation of the 3F3/2 phosphoepitope, thus, for SAC. While these results are interesting and provide novel information on the role of Polo and Mps1 kinases in SAC, there are many controls missing that would be required for these experiments and conclusions to be convincing.

Major concerns:

(1) The authors concluded that Polo activity is required for SAC signaling based on reduced levels of MCC (Figure 1). MCC formation is one of the indications of the SAC strength; however, MCC has also been shown to be formed independent of kinetochores, e.g. upon Mps1 promotion in cytosol as the authors suggested in the manuscript. Therefore, it is premature to make such a conclusion that Polo is required for SAC solely based on MCC formation without other evidence. It would be informative to examine the levels of Mad1/Mad2 on unattached kinetochores.

(2) There are no experiments showing the localization of the Ndc80 complex to kinetochores in the absence of Polo activity. The kinetochore-associated Ndc80 is necessary for the recruitment of Mps1 (Figures 2A and 3). Is Polo activity required for the kinetochore integrity in S2 cells?

(3) Although Polo inhibition de-localizes Aurora B from the inner centromere, the similar, if not more, intriguing phenotype is the increased levels of Aurora B at kinetochores (Figure 2B), which is not described or discussed in current manuscript. Do increased levels of Aurora B at kinetochores affect Aurora B-mediated phosphorylation of kinetochore components, kinetochore composition, and the strength of SAC?

(4) Without examining the levels of kinetochore-associated Ndc80 in Polo WT and T182D cells (Figure 3A), it is, again, difficult to determine whether it is the physical association of Ndc80 with the kinetochore or the Ndc80 phosphorylation by Polo and Aurora B kinases is important for the recruitment of Mps1 to unattached kinetochores.

(5) It would be informative to examine the Mad1/2 localization on metaphase kinetochores in the presence of T182D (Figure 3E) and anaphase onset with co-depletion of Mad2 (Figure 3G).

(6) The BubR1 was able to interact with Mps1 in cytosol, but remained unphosphorylated, and without Mps1 at the kinetochore, the cytosol BubR1 proteins detected by western blot analysis were not phosphorylated (Figure 4). Based on the current data, it is not clear whether kinetochore-associated BubR1 is phosphorylated or not in the absence of kinetochore-associated Mps1. An alternative explanation could be that Mps1 at the kinetochore is not necessary for BubR1 phosphorylation, but is essential for BubR1 dynamic association and dissociation with kinetochores. Without this dynamic turnover at kinetochores in the absence of Mps1, the majority of BubR1 proteins in cytosol remain unphosphorylated.

(7) The 3F3/2 phosphoepitope has been suggested to be the tension sensor. Does Polo T182D, which promotes Mps1 localization to bi-oriented kinetochores, also promote the 3F3/2 phosphoepitope signals on those metaphase kinetochores with tension?

(8) Over-expressing a kinase, Mps1, could cause many non-specific phosphorylations either in cytosol or on kinetochores. It is interesting to see increased levels of Mps1 on attached kinetochores in Mps1 over-expressing cells (Figure 7A), what about the Mps1 levels on unattached kinetochores?

(9) On one hand, the high levels of 3F3/2 phosphepitope and Cdc20 at kinetochores with normal attachment and tension might indicate that the SAC signaling cannot be satisfied in the presence of high levels of Mps1 (Figure 7B). It would be informative to examine the Mad1/2 levels. On the other hand, since cytosolic Mps1 can promote MCC formation independent of unattached kinetochores, it is not clear whether the higher levels of BubR1 with Cdc20 in Mps1 over-expressing cells is due to SAC signals on kinetochores or a large amount of Mps1 in cytosol to promote the kinetochore-independent BubR1-Cdc20 interaction (Figure 7C). It could provide some clarifications by examining the MCC formation in Ndc80-siRNA and Mps1-expressing cells.

Minor concerns:

The red, green, and blue labels in the figures are impossible to read and this could be improved by using a white background or moving the labels outside the figure panels.

1st Revision - authors' response 01 March 2013

Detailed answers to the reviewers

Referee #1 (Remarks to the Author):

Conde et al. address the functional relationship between the Polo and Mps1 kinases in the spindle assembly checkpoint (SAC) in Drosophila. They provide an extensive set of data from analyses mainly with cultured S2 cells and some with larval neuroblasts. They use established methodology. They demonstrate that

1. Polo depletion or addition of BI2536 result in a mitotic arrest that does not depend on the SAC proteins Mad2 and BubR1. Cdc20-IPs from the arrested cells have lower amounts of associated Mad2 and BubR1.

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5. Polo appears to have its effects on the intensity of 3F3/2 signals which are normally correlated with inter-sister kt tension indirectly via Mps1.

4. BubR1 phosphorylation but not kt localization depend on Mps1.

5. Mps1 appears to promote kt recruitment of Cdc20 by BubR1 and thereby promote formation of the complexes that inhibit the APC/C.

These findings are clearly novel in the Drosophila system. Most of these issues have previously been analyzed in human cells where the detailed functional characterization of the SAC is clearly more advanced than in Drosophila and where a far greater number of studies have been published, presumably because of the link to human cancer. Several findings by Conde et al. further emphasize that some mechanistic details of SAC function in humans and Drosophila are clearly different. The comparison between these two animal systems therefore points to the adaptive plasticity of the SAC. These differences should presumably be considered as a warning that SAC plasticity might also be displayed by different human cell types which not all might behave like HeLa cells. The eventual publication of this technically solid study will therefore be of interest for SAC research.

We thank the reviewer for the comments.

Apart from some minor problems, the following two appear to be major to me:

1. Conde et al are to be applauded for their comprehensive approach where they look at many SAC components. There is one obvious experiment that seems to be crucial for understanding the role of Polo and AurB for Mps1 kt localization. As also observed in experiments with human cells,

depletion or inhibition of these two kinases appears to have a partial effect on Mps1 kt localization. Human AurB for example is only required for a rapid association of Mps1 with the kt. Eventually, Mps1 levels are quite normal. Similarly, the manipulation of Polo and AurB preformed by Conde et al. do not abolish Mps1 kt localization completely. As also indicated in their model, these two kinases might act redundantly to some extent. This should be addressed by double depletion/inhibition experiments. These experiments will be important in clarifying how well we understand Mps1 kt localization.

We agree with the reviewer and, following his suggestion, we inhibited Aurora B in Polo depleted cells. This resulted in a much more dramatic effect on Mps1 kinetochore levels, which drop to 15% in colchicine treated cells (compared to 65% and 40% upon Aurora B inhibition and Polo depletion respectively). Our results suggest that these kinases are acting in concert to promote proper Mps1 kinetochore recruitment. In the revised manuscript we are now presenting this result in Figure 1 panel A and C.

2. The second major problem concerns the writing of the manuscript. In an attempt to integrate the vast SAC literature from vertebrates, Conde et al. mention various literature reports in a highly redundant fashion (often three times: in the introduction, in the result and the discussion section). As a result, the manuscript is very hard to read and not as concise as it should be. For example, Conde et al start the result section by stating that data concerning the role of Plk1 for SAC and 3F3/2 would be conflicting, and they continue "The conflicting data from vertebrate model systems prompted us to examine the role of Drosophila Polo in SAC signalling." I do not quite understand this logic. A conflict with data from vertebrate model systems cannot be resolved by looking at yet another system. Science does not work like democracy. (for another example see minor point 3) I am convinced that this manuscript would profit tremendously from extensive re-writing, aiming at a relatively short introduction (where the players are briefly introduced without unfolding details of disagreement) followed by a concise description of the experiments and the corresponding results (without debating their relation to the work in other systems) that are then discussed in relation to what has been found in other systems. With other words, the recommendation is to move most of the references concerning other systems, discrepancies and open questions to the discussion. Moreover, because this work clearly concerns Drosophila where the mechanistic details of SAC function are clearly not identical to all other eukaryotes, Conde et al should make this clear already in the title. In general, they should make it clearer what they think is of general validity and what might be Drosophila-specific. Some minor points to consider in this regard: Vleugel et al. 2012 have recently also pointed out interesting differences concerning the kinase domain in Drosophila BubR1. What do Conde et al. mean when they write about Cenp-E in Drosophila? Cenp-meta or Cenp-ana?

We have thoroughly revised the manuscript in order to remove redundancy and have, therefore, moved most of the comparisons with other systems to the discussion section. We have also altered the title partially to clarify that the work was done using *Drosophila* and have tried to make the distinction more clear between what we think is of general validity and what might be *Drosophila*-specific. Finally, Cenp-E refers to Cenp-meta, as indicated in page 11 of the revised version of the manuscript.

Minor points (order reflects appearance in text and not importance)
1. p.7: "Having established that Polo is required for the MCC formation, we ...": "is required for the" should be replaced by "promotes".

We agree with the suggestion and have replaced the sentence.

2. p.7: "... resulted in a significant reduction of the checkpoint kinase Mps1 at unattached kinetochores."
It is not clear to me how Conde et al. were able to decide in their experiments, whether a kinetochore is unattached or not. In a recent publication, the same lab has reported that in polo[9] mutants, chromosomes attach in a syntelic fashion. Therefore, just selecting cells where chromosomes are not neatly aligned in a metaphase plate is unlikely to assure that the kts are not attached.

Both S2 cells and *Drosophila* neuroblasts were treated with colchicine to guarantee that all kinetochores were unattached and, therefore, Mps1 levels at kinetochores were not affected by microtubule attachments.

3. p. 11: "Recent data in yeast and human cells showed that Mps1 phosphorylates KNL1/Spc105/Spc7 in its MELT repeats to promote direct binding of Bub3 to KNL1 thus allowing recruitment of Bub1 and BubR1 (London et al, 2012; Shepperd et al, 2012; Yamagishi et al, 2012)." and p22: "However, Drosophila Spc105/Knl-1 possesses species-specific MELT-like motifs that lack the phosphorylatable threonine but instead contain an excess of negative charges that possibly bypasses phosphodependency of recruitment controlled by Mps1 (Schittenhelm et al, 2009; Vleugel et al, 2012).
Schittenhelm et al. 2009 have demonstrated that BubR1 is still present at kts in Spc105 null mutants and also in internal deletion mutants lacking the "MELT"-repeats. Thus the suggestion that acidity of the Drosophila MELT-repeats seems to be an inappropriate speculation for explaining the discrepancy between Drosophila and humans. (As Schittenhelm et al. 2009 have made it already fairly clear that the role of Spc105 in BubR1 kt localization in Drosophila seems different from that in yeast and human cells, it makes little sense to mention the work in these alternative systems on p.11 before presenting the experiments concerning the role of Mps1 for BubR1 kt localization in Drosophila.)

We agree with the reviewer and have therefore removed these comments from the revised version of the manuscript.

4. It is quite striking that Mps1 IPs contain Ndc80 and BubR1 IPs do not (Fig. 4E). Moreover, Cdc20 IPs do not contain phosphorylated BubR1 (Fig. 7C). These results are not mentioned in the text. They are not really in line with the suggested model (Mps1 phosphorylates BubR1 at the kt which then can recruit Cdc20 efficiently). These observations need to be discussed. Similarly, why is phosphorylated BubR1 not apparent in Fig. 1D?

Our results indicate that Ndc80 is not required for BubR1 kinetochore recruitment in Drosophila (Figure 4A, B). Furthermore, our experiments suggest that Mps1 has to be recruited to kinetochores, where it presumably becomes fully active, in order to promote BubR1 phosphorylation. However, our experiments do not allow us to discriminate whether Mps1 interacts with BubR1 at kinetochores, or merely has to cycle off these structures to be able to do it, in which case these Mps1, BubR1 and Ndc80 may not exist in a single complex. Nevertheless, we cannot guarantee that such a complex does not exist, as it may simply not be detectable in our experimental conditions.

The apparent absence of phosphorylated BubR1 from Cdc20 IPs is an important question raised by the referee. Due to the high molecular weight of BubR1 (165 kDa), the slow migrating band is not consistently detectable in gels with more than 8% acrylamide, which accounts for it not being apparent in some immunoblots. To further clarify this we resolved BubR1 and Cdc20 IPs from control total cell lysates in 7% acrylamide gels with a ratio of acrylamide to bis-acrylamide of 80:1. This data, presented in Figure 6C in the revised manuscript, allows us to discern that Cdc20 co-immunoprecipitates mainly with the slow migrating isoform of BubR1. Therefore, it is our belief that the BubR1 detected in Cdc20 IPs is mostly phosphorylated.

5. p.18: "This markedly contrasts with cells that exclusively expressed endogenous Mps1, in which establishment of tension upon chromosome biorientation, precluded Mps1 localization at kinetochores and consequently resulted in a significant reduction of 3F3/2 and Cdc20 kinetochore levels (Figure 7A, B)."
As far as I understand, Fig 7A,B do not include data from cells that express exclusively endogenous Mps1. This data seems to be "not shown".

By stating "*cells that express exclusively endogenous Mps1"* we were referring to cells in which expression of EGFP-Mps1 was not induced. We agree and have, therefore, changed the text to "*non-induced cells*".

6. p.23: Conde et al discuss the role of Mps1 and Polo in generation of the 3F3/2 epitope. It would be of interest to reference Dou et al. 2011 which have suggested that these two kinases might have

largely overlapping substrate specificity.

The largely overlapping substrate specificity of the two kinases might suggest that Polo and Mps1 are both able to contribute to 3F3/2 independently of one another. However, our results clearly point to Polo being upstream of Mps1, as it is able to promote Mps1 kinetochore recruitment and affects its phosphorylation status, while the reverse is not supported by our results. Furthermore, Mps1 depletion caused a pronounced reduction of 3F3/2 phosphoepitope accumulation at kinetochores that stained positive for active Polo. Therefore, our work supports a model in which Polo promotes 3F3/2 generation by functioning upstream of Mps1. We preferred to withhold this reference to avoid confusion, which we deemed unnecessary.

7. p.23: "Importantly, constitutive kinetochores localization of Mps1 ...": It would be better to state here (as also later in the discussion) that Mps1 overexpression was used to enforce persistent kt localization. Mps1 fusions with kt localization signals to enforce constitutive localization in various systems have been reported but this is not what Conde et al. have done.

We thank the reviewer for pointing this out. We have amended to text to reflect this suggestion.

8. supplemental information: p.11 description of kt signal intensity quantification is not clear. Individual kts are said to have been selected manually, but later in the sentence a ROI is mentioned that includes all kts. Please clarify.

We thank the reviewer for pointing this out. We have altered the text to clarify that kinetochores were selected individually and the size of the ROI was predefined so that each single kinetochore would fit into it.

9. typographical errors:
Fission -> fission (p. 16)
occurs -> occur (p. 19)
hyperphosphorytated (p. 24)
neuroblats (p. 36)
in Suppl. information:
describe -> described (p. 11)
ressuspended, Mercaptoetanol, Glicerol (p. 12)
p. 13: does the "(x)" after anti-Fizzy/Cdc20 and anti-Cdc27 need to be there?

All these typographical errors and have been corrected

Referee #2 (Remarks to the Author):

The manuscript entitled "Polo regulates the SAC through MPS1-dependent BubR1 phosphorylation" presents very well documented new findings exploring the interplay between Aurora B, MPS1, the Ndc80/Nuf2 complex, and Polo. However, in my opinion, some of the data are over-interpreted, which precludes from ending with a clear picture of this interplay. Nevertheless, my concerns are more about the writing than the data.

1- As a major breakthrough, Conde et al. show that in the absence of Polo protein or kinase activity, cells are delayed in metaphase independently of Mad2 or BubR1. As the authors propose, this could reflect an essential role for Polo in regulating the APC/C. Of course, it would have added huge value if they could have demonstrated it directly. I assume that the authors failed to measure APC/C activity onto Cdc27 immunoprecipitates (for which they have antibodies, as shown in Fig. 6D), but if this is not the case, they should definitely try this experiment.

We agree that it would be very interesting to further evaluate a possible role for Polo in APC/C regulation. In fact, we think that this can be a very interesting project on its own. Nevertheless, following up on the reviewer's question, we tried to ascertain whether ubiquitination was hampered in the absence of Polo. To this end, we immunoprecipitated EGFP-Cyclin B and evaluated the extent of its ubiquitination using the FK2 antibody to detect ubiquitinated conjugates. Our results indicate

that Polo is in fact required to promote proper Cyclin B ubiquitination, which supports the hypothesis that Polo regulates APC/C activity. The results are now included in Figure 1D in the revised manuscript.

The authors then ask whether Polo could also have a role in controlling the checkpoint. They show that the formation of the APC/C inhibitor (MCC: Cdc20-BubR1-Mad2 complex) is impaired in Polo defective cells. This finding argues that the arrest observed upon Polo deficiency might indeed be independent of the checkpoint, since the formation of MCC is partially impaired in this condition. Showing that Mad2 is not recruited to the kinetochores in the absence/inhibition of Polo would definitely strengthen the point that the arrest is independent from the SAC (without this easy experiment, one can still wonder if Polo could have a role in silencing an even weakened checkpoint, for instance).

We agree with the reviewer and have, therefore, added a panel with the levels of Mad2 at unattached kinetochore in control cells and upon Polo depletion and inhibition. The results, now included in Figure 1F of the revised manuscript, show decreased Mad2 levels upon Polo depletion and inhibition, which strengthens the importance of Polo in proper SAC signaling, as well as the point that the arrest in its absence/inhibition is indeed SAC independent.

Indeed, it is unclear to me why they need to overactivate the checkpoint with colchicine to demonstrate that Polo deficiency arrests the cells in metaphase independently of the checkpoint. If Polo is required to activate the APC/C, why do we need to activate the checkpoint to uncover it?

The mitotic index upon colchicine treatment makes it clear that BubR1 and Mad2 were depleted to such an extent that it did, in fact, abrogate SAC function. However, following up on the reviewer's question we have added a graph with the mitotic indices in asynchronous cultures, which strengthens the point that Polo depletion or inhibition results in a mitotic arrest. The data is now included in Figure 1A of the revised manuscript.

2- The authors clearly show that upon Polo inhibition/reduction, MPS1 kinetochore recruitment and overall phosphorylation levels are reduced, which could explain why less MCC is produced upon Polo inhibition/reduction. However, the concept that MPS1 could auto-activate upon dimerization and autophosphorylation at the kinetochore was proposed (Kang et al., 2007; Jelluma et al., 2008), but not demonstrated, and is not the sole mechanism. It has also been shown that MPS1 is activated via CDK1-dependent phosphorylation, independently of the kinetochore (Morin et al., Curr. Biol., 2012). The authors should not ignore that we still know little about MPS1 mode of activation. What we do know, is that MPS1 is heavily phosphorylated, but showing a reduction in the intensity of lower-migrating bands, (i) does not mean lack of phosphorylation at the kinetochore, and (ii) does not necessarily mean lack of activity. Indeed, some phosphorylation(s) could be inhibitory, and hyperphosphorylation (directly by Polo for instance?) often leads to a smear in the migration pattern which can be misinterpreted as a diminution of phosphorylation (faster migrating forms will diminish the intensity of each individual band). In other words, to claim that they are monitoring MPS1 activity by looking at its phosphorylation, the authors should use a phospho-specific antibody known to detect an activatory phosphorylation. Otherwise, they can just claim that MPS1 phosphorylation is affected in absence of Polo activity, which is already interesting.

Following up on the reviewer's comment, as it has been described that Mps1 auto-phosphorylates at kinetochores to achieve full kinase activity, we tried to evaluate autophosphorylation using an antibody specific for Mps1 T676 phosphorylation (Jelluma *et al*., 2008). However it failed to work in immunoblots of *Drosophila* total cell lysates. We agree with the reviewer when he states that a phosphorylation does not always reflect activation. However in what concerns Mps1 this has been a common approach in previous publications. Nervertheless we have tried to alter the text to reflect this suggestion.

3- Using an Aurora B phospho-mimicking Polo mutant (Polo-T182D), the authors elegantly demonstrate that Polo is required upstream of MPS1, favoring its recruitment at the kinetochore, independently of Aurora B. They further show that, on the contrary, MPS1 recruitment at kinetochores is dependent on Ndc80/Nuf2. To strengthen this point, they used high-resolution imaging to show that Polo-T182 phosphorylation and MPS1 are concomitantly lost upon increase of

the interkinetochore distance (and concomitant escape from Aurora-B dependent phosphorylation). Strikingly, Polo-T182 constitutive phosphorylation is sufficient to recruit MPS1 onto these distant, correctly attached, late prometaphase kinetochores. This really argues for a direct role of Polo in recruiting MPS1 at the kinetochore in drosophila.

4- Another well documented finding is the role for MPS1 in controlling BubR1 & 3F3/2 hyperphosphorylation at the kinetochore (but not BubR1 recruitment to the kinetochore). In sharp contrast with previous studies, the fact that the 3F3/2 staining disappears upon Nuf2/Ndc80 depletion (which prevents MPS1, but not Polo recruitment at the kinetochore) argues for MPS1 being a more direct kinase than Polo to generate the 3F3/2 epitope.
Then, they show that in addition to members of the Aurora B Polo -> Ndc80/Nuf2 -> MPS1 -> P-BubR1 pathway, Bub3 is also required to recruit Cdc20 at the kinetochore. Altogether, this suggests that phospho-BubR1 is an important player of Cdc20 recruitment at the kinetochore. This is further supported by the results showing that upon artificial maintenance of MPS1 -> P-BubR1/3F3 at kinetochores in late prometaphase (when kinetochores are attached and stretched), -> Cdc20 is also maintained at kinetochores.
The authors finally show that EGFP-Mps1 is required to stabilize the formation of the Cdc20-BubR1 complex in the cytoplasm, but that this does not sustain the checkpoint as efficiently as in the presence of kinetochore-dependent catalysis, confirming previous findings obtained in human cell cultures (Maciejowski et al., 2010).

Various issues:
- I have a concern regarding the use of the Polo9 mutant, described in Donaldson et al., J. Cell Biol., 2001. In this publication (that the current manuscript forgets to cite), they were claiming that Polo deficiency was causing a metaphase arrest with centromere separation. This discrepancy of phenotype should be discussed in the current manuscript. I am not aware of any follow-up on this "metaphase arrest with centromere separation" phenotype, but I would be glad to know.

We thank the reviewer for pointing this out and have added this reference. However, the discrepancy of phenotype has already been discussed in a previous paper from our lab – Moutinho-Santos et al., 2012. It is our feeling that discussing this in the present work would not benefit our work substantially.

- The sentence p.9: "(...) and suggest that Polo may control MPS1 recruitment to kinetochores indirectly by promoting the correct localization and activation of Aurora B" is misleading since the paper later demonstrates that this is not the case. The sentence could stop before the "and" without missing anything.

We agree and have amended the text as suggested in this comment.

- *The legends embedded in the immunofluorescence images are very difficult to read on screen, and impossible to read once printed (although the figures are still at full scale). These labels should be enlarged and bolded, and/or printed on white layer, or indicated above the figure.*

We thank the reviewer for pointing this out. We have altered the labels accordingly.

- *p.10; p.14, ...: it should be mentioned whether they are referring to inter- or intra-kinetochore tension.*

We thank the reviewer for pointing this out. In our experiments, we only evaluated interkinetochore distance. We have altered the text to clarify this.

Referee #3 (Remarks to the Author):

Review of Conde et al, EMBOJ-2012-83630

Polo regulates the spindle assembly checkpoint through Mps1-dependent BubR1 phosphorylation

Conde and colleagues investigated the role of Polo kinase in the spindle assembly checkpoint in Drosophila cells. They demonstrated that the active Polo kinase is essential for kinetochore recruitment of Mps1. They subsequently showed that Mps1 kinetochore localization is required for BubR1 hyperphosphorylation and formation of the 3F3/2 phosphoepitope, thus, for SAC. While these results are interesting and provide novel information on the role of Polo and Mps1 kinases in SAC, there are many controls missing that would be required for these experiments and conclusions to be convincing.

Major concerns:

(1) The authors concluded that Polo activity is required for SAC signaling based on reduced levels of MCC (Figure 1). MCC formation is one of the indications of the SAC strength; however, MCC has also been shown to be formed independent of kinetochores, e.g. upon Mps1 promotion in cytosol as the authors suggested in the manuscript. Therefore, it is premature to make such a conclusion that Polo is required for SAC solely based on MCC formation without other evidence. It would be informative to examine the levels of Mad1/Mad2 on unattached kinetochores.

We agree that it would be informative and have added Figure 1F of the revised manuscript with the levels of Mad2 on unattached kinetochores in control, Polo-depleted and BI-treated cells. We do find that Polo depletion or inhibition has a profound effect upon Mad2 kinetochore recruitment and this has now been discussed in the relevant section of the manuscript.

(2) There are no experiments showing the localization of the Ndc80 complex to kinetochores in the absence of Polo activity. The kinetochore-associated Ndc80 is necessary for the recruitment of Mps1 (Figures 2A and 3). Is Polo activity required for the kinetochore integrity in S2 cells?

We agree that this is a very interesting point and following this suggestion we have evaluated Ndc80 levels at kinetochores in the different experimental conditions. In fact, our results indicate that Polo is responsible for 50% of Ndc80 kinetochore levels and this may partially explain the reduction of Mps1 at kinetochores. This data has been included in Figure 2B-C and the relevance of this observation has now been fully discussed in the revised manuscript.

(3) Although Polo inhibition de-localizes Aurora B from the inner centromere, the similar, if not more, intriguing phenotype is the increased levels of Aurora B at kinetochores (Figure 2B), which is not described or discussed in current manuscript. Do increased levels of Aurora B at kinetochores affect Aurora B-mediated phosphorylation of kinetochore components, kinetochore composition, and the strength of SAC?

We agree that this is in fact a very interesting point, which we are currently trying to evaluate. However it is beyond the scope of the current work.

(4) Without examining the levels of kinetochore-associated Ndc80 in Polo WT and T182D cells (Figure 3A), it is, again, difficult to determine whether it is the physical association of Ndc80 with the kinetochore or the Ndc80 phosphorylation by Polo and Aurora B kinases is important for the recruitment of Mps1 to unattached kinetochores.

We agree that this is an important point and have, therefore, evaluated Ndc80 kinetochore levels in these cell lines. This data has been included in Figure 3A-B of the revised manuscript. Constitutively active Polo was able to restore Ndc80 kinetochore levels upon Aurora B inhibition, further reinforcing the role of Polo in outer kinetochore assembly. As this correlates with a recovery of Mps1 kinetochore levels, it supports the hypothesis that Polo promotes proper Mps1 kinetochore localization at least in part by promoting Ndc80 kinetochore association. However, it does not explain how active Polo is able to maintain Mps1 at kinetochore pairs, which are under tension, as evaluated by the interkinetochore distance.

(5) It would be informative to examine the Mad1/2 localization on metaphase kinetochores in the presence of T182D (Figure 3E) and anaphase onset with co-depletion of Mad2 (Figure 3G).

We agree that it would be informative to do so and are currently working on this. We have evaluated the effect of Mad2 depletion in a cell line expressing EGFP-Polo^{T182D} on anaphase onset and verified that, as expected, it also shortens the time between NEB and anaphase onset. This data is now included in Figure 3H of the revised manuscript.
Although adding information about Mad1/2 localization on metaphase kinetochores in the same cell line is a valuable suggestion, we do not feel that it would add substantial information to this manuscript. Further work on this aspect is in progress in the laboratory.

(6) The BubR1 was able to interact with Mps1 in cytosol, but remained unphosphorylated, and without Mps1 at the kinetochore, the cytosol BubR1 proteins detected by western blot analysis were not phosphorylated (Figure 4). Based on the current data, it is not clear whether kinetochore-associated BubR1 is phosphorylated or not in the absence of kinetochore-associated Mps1. An alternative explanation could be that Mps1 at the kinetochore is not necessary for BubR1 phosphorylation, but is essential for BubR1 dynamic association and dissociation with kinetochores. Without this dynamic turnover at kinetochores in the absence of Mps1, the majority of BubR1 proteins in cytosol remain unphosphorylated.

We agree that this is a very interesting suggestion. To clarify this issue we have performed FRAP experiments to evaluate BubR1 dynamics in control and Mps1-depleted cells. Our results indicate that BubR1 dynamics fits a double exponential curve with a fast and a slow phase. Furthermore, in the absence of Mps1 BubR1 was still able to dynamically exchange at kinetochores. The results have now been included in Figure 4C and the relevance of this observation is further discussed in the manuscript.

(7) The 3F3/2 phosphoepitope has been suggested to be the tension sensor. Does Polo T182D, which promotes Mps1 localization to bi-oriented kinetochores, also promote the 3F3/2 phosphoepitope signals on those metaphase kinetochores with tension?

We feel that this is an important experiment to strengthen our conclusions. A careful evaluation of 3F3/2 levels at bi-oriented kinetochores in EGFP-Polo^{T182D} expressing cells supports that active Polo is able to promote 3F3/2 labeling at metaphase kinetochores. The data is included now in Figure 5E.

(8) Over-expressing a kinase, Mps1, could cause many non-specific phosphorylations either in cytosol or on kinetochores. It is interesting to see increased levels of Mps1 on attached kinetochores in Mps1 over-expressing cells (Figure 7A), what about the Mps1 levels on unattached kinetochores?

Our *in vivo* and IF analysis revealed that when overexpressed EGFP-Mps1 maintained the ability to localize at kinetochores from early prometaphases. The ability to localize at unattached kinetochores was further confirmed in fixed cells treated with colchicine (data not shown).

(9) On one hand, the high levels of 3F3/2 phosphepitope and Cdc20 at kinetochores with normal attachment and tension might indicate that the SAC signaling cannot be satisfied in the presence of high levels of Mps1 (Figure 7B). It would be informative to examine the Mad1/2 levels. On the other hand, since cytosolic Mps1 can promote MCC formation independent of unattached kinetochores, it is not clear whether the higher levels of BubR1 with Cdc20 in Mps1 over-expressing cells is due to SAC signals on kinetochores or a large amount of Mps1 in cytosol to promote the kinetochore-independent BubR1-Cdc20 interaction (Figure 7C). It could provide some clarifications by examining the MCC formation in Ndc80-siRNA and Mps1-expressing cells.

We agree that these are interesting points. Following the reviewer's suggestions, we evaluated Mad1 levels at metaphase kinetochores in cells overexpressing Mps1. This data is included in Supplementary Figure S5E. Our quantifications reveal that localization of Mad1 on metaphase kinetochores is increased upon Mps1 overexpression when compared to non-induced cells.
We also agree that it would be interesting to further evaluate the role of cytosolic Mps1. However, under overexpression conditions, depletion of Ndc80 does not preclude Mps1 localization (unpublished data). This likely reflects unspecific binding of overexpressed Mps1 to kinetochores that does not occur under normal expression conditions. Consequently we were unable to further evaluate this issue as suggested by the reviewer. Further work would be needed to better assess the role o cytosolic mps1.

Minor concerns:

The red, green, and blue labels in the figures are impossible to read and this could be improved by using a white background or moving the labels outside the figure panels.

We thank the reviewer for pointing this out and have adjusted the figures accordingly.

2nd Editorial Decision 20 March 2013

Thank you for submitting your revised manuscript for our consideration. Two of the original reviewers have now reviewed it once more (see comments below), and I am pleased to inform you that they have no more principle concerns regarding publication in The EMBO Journal. We shall therefore be happy to accept your study once a few minor (editorial) issues have been taken care of:

- Please modify the text to incorporate the remaining minor points of referee 1.

- Referee 3 also retains some concerns, which in my view would be appropriately addressed by additional clarifications and qualifications in the text, as well as by mentioning potential alternative explanations in the discussion.

Once we will have received the adequately re-revised version, we should then be able to swiftly proceed with formal acceptance and production of this paper. Let me know if you should have any further questions.

I look forward to receiving your final version.

———

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

To arrive at their revised version, Conde et al. have performed additional experiments and have re-written much of the original text. These measures have certainly led to significant improvements. They have clearly solved the major problems that I have detected in the original version. Moreover, according to my perception, they have also addressed all major comments of the other referees in a satisfactory manner.

It should be straightforward to solve the following remaining problems:

1. p.11: "... or CENP-meta (CENP-E orthologue)"
The Drosophila CENP-meta and CENP-ana genes have resulted from a relatively recent head to head gene duplication. In case of CENP-meta some functional characterization has been published. However, CENP-ana remains largely uncharacterized. There is no clear evidence which of these two paralogs is functionally more similar to CENP-E. Given these facts, it is misleading to declare CENP-meta to be the CENP-E ortholog. Most appropriate therefore would be "... or CENP-meta (one of two Drosophila CENP-E paralogues)".

2. p.24: "Importantly, constitutive kinetochore localization of Mps1 ..."
While Conde et al. have changed their original wording in other passages, here they still generate the misleading impression (see may comments to the original version) that they have enforced constitutive kinetochore localization.

3. As indicated in my comments on the original version, in the interest of clarity it is crucial to be clear about concordant and discordant features of the SAC in vertebrates and Drosophila. This

necessitates that also the findings of Suijkerbuijk et al. (2012) with regard to BubR1 protein kinase activity are stated in this manuscript. Accordingly, Drosophila BubR1 has intact kinase features in contrast to vertebrates where BubR1 is a pseudokinase. In addition, these findings of Suijkerbuijk et al. (2012) should also be included more explicitly to raise an important caveat about the work of Guo et al. (2012) so that not just one side of a controversy is represented.

4. p. 11 "notorious" -> "noticeable"

5. p. 36 (legend Fig 3): "yellow *"
I think that there is no "yellow *" in the figure (Sorry for not having pointed this out earlier).

Referee #3 (Remarks to the Author):

This current manuscript by Conde et al is improved as compared to the previous submission. The authors have addressed many concerns, both from this reviewer and two other reviewers, in a number of instances with new experiments. Data presented in the current manuscript support a role for Mps1 kinetochore localization in BubR1 hyperphosphorylation and formation of the 3F3/2 phosphoepitope. These results are interesting and provide novel information on the role of Mps1 in SAC.

Based on the newly added data on the requirement of Polo on Ndc80 kinetochore localization as I suggested in the previous review, the original doubts that I expressed remain that the role of Polo on Mps1 kinetochore localization is largely indirect. The Polo, along with the Aurora B activity, clearly has a profound role in outer kinetochore assembly. The further reduction of levels of kinetochore-associated Mps1 with dual inhibition of Polo and Aurora B could still be attributed to an indirect effect on outer kinetochore assembly, though independent of Ndc80. Although active Polo is able to maintain Mps1 at kinetochore pairs with tension, how a significantly dropped level of kinetochore-associated active Polo (PoloT182Ph) (Figure 3, D and F) is able to promote Mps1 localization remains unknown. This could be due to a direct phosphorylation event, but this is not demonstrated in the manuscript.

The data presented in newly added Figure 1D are confusion. First, in the figure legend, it is stated cells were treated with MG132 for 2 h; however, it is not known whether there is any other treatment, e.g. nocodazole to increase the percentage of mitotic cells, prior to MG132 treatment. And second, it is not clear why the input levels of EGFP-Cyclin B and Cyclin B differs, assuming this represents the protein level in cells. It seems that the levels correlated to Mad2 and BubR1 siRNA (but not Polo), but why? If it's not due to protein degradation (MG132), is it because of ubiquitination? However, this would be inconsistent with a potential role for Polo in APC/C activation as the author suggested.

2nd Revision - authors' response 20 April 2013

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To arrive at their revised version, Conde et al. have performed additional experiments and have re-written much of the original text. These measures have certainly led to significant improvements. They have clearly solved the major problems that I have detected in the original version. Moreover, according to my perception, they have also addressed all major comments of the other referees in a satisfactory manner.

It should be straightforward to solve the following remaining problems:

1. p.11: "... or CENP-meta (CENP-E orthologue)"
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We agree with the reviewer suggestion and have replaced the sentence accordingly.

2. p.24: "Importantly, constitutive kinetochore localization of Mps1 ..."
While Conde et al. have changed their original wording in other passages, here they still generate the misleading impression (see may comments to the original version) that they have enforced constitutive kinetochore localization.

We thank the reviewer for pointing this out once again. We have amended the text to prevent misleading impressions.

3. As indicated in my comments on the original version, in the interest of clarity it is crucial to be clear about concordant and discordant features of the SAC in vertebrates and Drosophila. This necessitates that also the findings of Suijkerbuijk et al. (2012) with regard to BubR1 protein kinase activity are stated in this manuscript. Accordingly, Drosophila BubR1 has intact kinase features in contrast to vertebrates where BubR1 is a pseudokinase. In addition, these findings of Suijkerbuijk et al. (2012) should also be included more explicitly to raise an important caveat about the work of Guo et al. (2012) so that not just one side of a controversy is represented.

We agree with the referee's reasoning and have therefore stated in the discussion of the manuscript (p.23 of the revised version) the findings published by Suijkerbuijk et al, (2012) and highlighted the differences between vertebrate and *Drosophila* BubR1.

4. p. 11 "notorious" -> "noticeable"
We thank the reviewer for pointing this and have amended the text.

*5. p. 36 (legend Fig 3): "yellow *" - I think that there is no "yellow *" in the figure (Sorry for not having pointed this out earlier).*

We thank the reviewer for the comment. In Figure 3, panel D, we included a yellow * to highlight a misaligned chromosome.

Referee #3 (Remarks to the Author):

This current manuscript by Conde et al is improved as compared to the previous submission. The authors have addressed many concerns, both from this reviewer and two other reviewers, in a number of instances with new experiments. Data presented in the current manuscript support a role for Mps1 kinetochore localization in BubR1 hyperphosphorylation and formation of the 3F3/2 phosphoepitope. These results are interesting and provide novel information on the role of Mps1 in SAC.

Based on the newly added data on the requirement of Polo on Ndc80 kinetochore localization as I suggested in the previous review, the original doubts that I expressed remain that the role of Polo on Mps1 kinetochore localization is largely indirect. The Polo, along with the Aurora B activity, clearly has a profound role in outer kinetochore assembly. The further reduction of levels of kinetochore-associated Mps1 with dual inhibition of Polo and Aurora B could still be attributed to an indirect effect on outer kinetochore assembly, though independent of Ndc80. Although active Polo is able to maintain Mps1 at kinetochore pairs with tension, how a significantly dropped level of kinetochore-associated active Polo (PoloT182Ph) (Figure 3, D and F) is able to promote Mps1 localization remains unknown. This could be due to a direct phosphorylation event, but this is not demonstrated in the manuscript.

To address the referee's concern regarding a possible indirect effect on outer kinetochore assembly (Ndc80-independent) upon simultaneous loss of Polo and Aurora B activities, we provide this possibility as an alternative explanation for the observed impairment of Mps1 kinetochore recruitment in the current version of text (p. 9, line 6, 1st paragraph). Nevertheless, this does not preclude the existence of other mechanisms, through which Polo may be controlling Mps1 kinetochore localization, either in a direct or indirect fashion. In fact, the decrease of Mps1 levels at kinetochore pairs under tension correlates with a decrease in PoloT182Ph staining and conversely expression of constitutively active Polo is able to promote the recruitment of Mps1 to kinetochores of metaphase chromosomes. We therefore argue that Polo regulates Mps1 kinetochore localization at multiple levels but a direct regulation was never claimed in the present manuscript. We are presently working to uncover the molecular mechanisms by which Polo promotes outer kinetochore assembly and Mps1 recruitment.

The data presented in newly added Figure 1D are confusion. First, in the figure legend, it is stated cells were treated with MG132 for 2 h; however, it is not known whether there is any other treatment, e.g. nocodazole to increase the percentage of mitotic cells, prior to MG132 treatment. And second, it is not clear why the input levels of EGFP-Cyclin B and Cyclin B differs, assuming this represents the protein level in cells. It seems that the levels correlated to Mad2 and BubR1 siRNA (but not Polo), but why? If it's not due to protein degradation (MG132), is it because of ubiquitination? However, this would be inconsistent with a potential role for Polo in APC/C activation as the author suggested.

As stated in the respective figure legend, cultured cells were incubated with MG132 without any additional drug. We share the referee's doubts in what regards EGFP-Cyclin B and Cyclin B levels found in cell lysates. This can be partially explained by different amounts of total protein loaded in the gel, as reflected by the tubulin levels. We have therefore repeated the experiment using new extracts. The results are now included in Fig.1D and show that cyclin B levels indeed increase upon Polo depletion. This likely reflects the corresponding increase of the mitotic index rather then the observed reduction of Cyclin B ubiquitination given that for all RNAi conditions cells were treated with MG132 to prevent protein degradation. Analysis of ubiquitinated EGFP-cyclin B levels correlated with the presence or absence of Polo kinase and therefore APC/C activity, fully confirming our previous results.

Acceptance letter 23 April 2013

Thank you for submitting your revised manuscript for our consideration. I am pleased to inform you that following your final modifications, we are now able to accept it for publication in The EMBO Journal.

