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Xenopus Shugoshin 2 regulates the spindle assembly pathway mediated by the chromosomal passenger complex

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1st Editorial Decision 09 August 2011

Thank you for submitting your manuscript on XSgo2 for our consideration. Three experts have now reviewed it, and I am pleased to inform you that all of them consider your results of interest and in principle suited for publication in The EMBO Journal. They nevertheless raise several important concerns that would need to be addressed prior to publication. Given that these criticisms appear to be well-taken, and mostly pertain to the presented data and conclusion (rather than to further-reaching aspects), I would like to give you the opportunity to respond to them in the form of a revised version of the manuscript.

In this respect, it will be important to address key technical points (e.g. ref 1 pt 2, ref 2 pt 5 & 6, ref 3 pt 1, 2) but also the criticisms regarding experimental conclusiveness (e.g. ref 1 pt 1, ref 2 pt 3 ref 3 pt 5 relating to PP2A, ref 2 pt 1 and ref 3 pt 4 relating to MCAK, ref 2 pt 2 and 4). Please also re-consider the representation and statistical analysis of numerical data (as also requested by referee 1) - in this respect please note no statistically meaningful errors can be calculated for samples with N=2; in such cases we would require either an increase in the number of experimental replicates (N{greater than or equal to}3) to allow for proper statistical analysis, or a re-plotting of these data to show individual data points in vertical arrangement instead of a column plot. Finally, when revising the manuscript text, please make sure to amend it with a brief author contribution statement and conflict of interest declaration.

Pending adequate revision, we should be happy to consider the study further for publication. Please note however that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version. When preparing your letter of response to the referees' comments, please also bear in mind 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:

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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.

Please do not hesitate to contact me in case you should have any additional question regarding this decision. I look forward to your revision.

Yours sincerely,
Editor
The EMBO Journal

———

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

Shugoshins are important regulators of chromosome segregation and have two major functions attributed to them: the protection of centromere cohesion and sensing of tension between sister kinetochores. Most organisms have two Sgo proteins, but there is not yet a clear consensus of the exact contribution of the Sgo1 and Sgo2 proteins to the specific functions of Shugoshins. This manuscript describes the identification and characterization of Xenopus Sgo2 (XSgo2). XSgo1 was previously identified and shown to be required for the protection of centromeric cohesion. Here the authors show that XSgo2 does not participate in the protection of centromeric cohesion, but that it is required for proper bipolar spindle formation and chromosome alignment. They show evidence that XSgo2 is not required for the localization of the Chromosome passenger complex at centromeres, but that it might be required for its full activation in the extracts. Because the CPC has previously been demonstrated to be required for proper spindle assembly in this system, this could offer an explanation for the spindle phenotypes of XSgo2-depleted extracts. Finally, the authors show that XSgo2 pulls down a different PP2A holoenzyme than XSgo1.

This is a very nice characterization of Xenopus Sgo2 protein. Although much has been published on Sgo proteins in general, a clear consensus has not emerged and further information from the Xenopus system is very valuable. The paper is extremely well written, gives a good overview of the background and the quality of the data is high. The paper could however be strengthened by the following modifications:

1. The authors demonstrate nicely that XSgo2 associates with the B56 subunit-containing PP2A and propose that the specific functions of XSgo2 in spindle function are mediated through this form of PP2A. What is the effect of depletion of B56 on spindle assembly in these extracts. It would greatly strengthen the paper to show some data to support this hypothesis.

2. The conclusion that depletion of XSgo2 decreases Aurora B activity is based on the kinase assay shown in Figure 6C. To make this conclusion this experiment should be more rigorous. Are the cellular levels of Aurora B the same in the three conditions? The percentages of activity should be shown for three independent experiment repeats with an indication of the error.

3. Some experiments show error bars even though only two experiments are included. E.g. Fig. 2B and S3.

Referee #2 (Remarks to the Author):

Xenopus Sgo2 regulates the spindle assembly pathway mediated by the CPC

Rivera et al.

Accurate chromosome segregation requires that chromosomes bi-orient on the mitotic spindle. The presence of centromeric cohesin as well as regulators of kinetochore-microtubule attachments, such as Aurora B kinase, are essential for this. The Shughosin proteins protect centromeric cohesin and control the localization of the chromosomal passenger complex thereby contributing to bi-orientation and the proper segregation of sister-chromatids in anaphase. This study describes the identification of Xenopus Sgo2 and dissects the function of XSgo1 and XSgo2 in this model organism. While XSgo1 controls centromeric cohesion, this study shows that XSgo2 is not involved in the protection of centromeric cohesin but instead regulates Aurora B kinase activity, MCAK localization, spindle assembly and chromosome alignment. Moreover, the two XSgo proteins interact with different PP2A regulatory subunits proposed to dictate their non-overlapping functions.

Overall this is an interesting study on an important group of proteins, but at the moment a number of the conclusions are not evidence based. Below I have listed the points that need to be improved.

1) The authors conclude that the "presence of an abnormally large fraction of active MCAK depolymerase along chromosome arms" would hinder spindle assembly in the absence of XSgo2 (p.11). The experiments shown in figure 5 do not convincingly show this, and the authors should demonstrate that the effect of Sgo2 depletion on spindle assembly is indeed dependent on MCAK. Co-depletion of MCAK and XSgo2 is expected to revert the spindle assembly defect (fig 3). This experiment should be done.

2) The authors suggest that the decrease in Aurora B kinase activity (fig. 6) seen after XSgo2 depletion is responsible for the spindle assembly defects, but this is not formally shown. The phenotype of Sgo2 depletion is not as strong as for CPC depletion (p. 15), and in the discussion the authors provide a plausible explanation for this: Sgo2 and Haspin may act together in activating Aurora B and hence depletion of one still allows some activity by the other. This translates into an easy experiment (codepletion of Haspin and XSgo2 should enhance the functional defects shown in fig 3) that would strengthen the message of the paper.

3) In figure 7 it is demonstrated that XSgo1 and XSgo2 interact with different regulatory subunits of PP2A. This is potentially very interesting but unfortunately no evidence is provided that this differential interaction relates to the different functions of the two Sgo proteins, as presented in the model of figure 8. The authors should provide at least some evidence that this might be the case. B56 and B56 should be depleted and the consequence on centromeric cohesin and on Aurora B localization and activity (pT248) should be determined.

4) The finding that XSgo1 depletion results in a dramatic decrease in centromere localized Aurora B, but hardly affects centromeric T248 phosphorylation of Aurora B is puzzling and intriguing but not explained. It suggests that the very small pool of Aurora B that remains at the centromere after Sgo1 depletion is highly active and that Sgo1 may thus have an inhibitory effect on the activation of Aurora B. Since Sgo2 promotes Aurora B activation (pT248), the following scenario might be the case: While Sgo1 promotes the centromeric recruitment of Aurora B, at the same time it also inhibits its kinase activity, but this inhibitory effect is on its turn counteracted by Sgo2. If true, then it is expected that in the absence of Sgo1, Sgo2 depletion will no longer affect T248 phosphorylation of Aurora B nor the centromeric localization of MCAK. In other words, the authors should show what happens to the centromeric pT248 and MCAK levels in the XSgo1/XSgo2 double depleted cells. If it is similar to the XSgo1 single depleted cells and thus higher than in the XSgo2 single depleted cells, then this could be an explanation for how the two Sgo proteins locally control CPC activity. If the XSgo1/XSgo2 double depletion is similar to the XSgo2 single depletion, then the explanation given on p. 17 is more likely.

5) Fig. 6A: The control antibody (RbIgG) immunoprecipitates lots of INCENP and Dasra A (lane 2), while it does not bind PPA2A-C or PP2A-B56 (fig 7C). This hampers the conclusion that XSgo1 and XSgo2 interact with the CPC as it could be explained by aspecific binding to the rabbit IgG.

6) Fig 6C: In contrast to figure 6D, this is not a convincing experiment to show that Sgo2 promotes Aurora B kinase activity. First, there is no evidence that the CBB stained band in the upper panel is indeed INCENP. Second, the reduction in phospho-INCENP seen in the Sgo2 depleted cells can be

explained by the reduction in the amount of INCENP (i.e. the CBB band is less intense compared to the other two bands).

7) Fig. 4B: In human cells depletion of Sgo1 chromosome alignment is severely disrupted (Salic, Cell 2004, McGuinness et al., Plos Biol 2005) due to loss of centromere cohesion. Do the authors have an explanation for the fact that this is not seen in Xenopus extracts (fig. 4)?

Minor points:
Fig. 1B: Please show insets with magnifications of the kinetochores , to show that XSgo1 and XSgo2 have overlapping localizations in mitosis.

p. 5: "at least vitro", should be "at least in vitro"

p. 31 legend figure 7: "Tale 1" should be "Table 1"

Referee #3 (Remarks to the Author):

In this interesting manuscript, Rivera et al. use frog egg extracts to study the function of Xenopus Sgo2. Surprisingly, and unlike XSgo1, XSgo2 does not regulate sister chromatid cohesion but instead influences meiotic spindle assembly. Extracts depleted of XSgo2 form monopolar structures or spindles that have reduced microtubule levels, an effect attributed to enhanced chromosome arm localization of active MCAK. XSgo2 interacts with the CPC and PP2A complexes, which leads the authors to propose that XSgo2 functions in the CPC arm of the spindle assembly pathway. XSgo2 is suggested to contribute to CPC activation, which would block MCAK activity during spindle assembly and thus promote microtubule polymerization.

Major points:
1. A major concern about the XSgo2 depletion studies is that a rescue experiment is not performed. If XSgo2 is difficult to obtain in recombinant form, is it possible to perform an add-back using the in vitro transcription/translation approach? This data seems particularly important given that XSgo2 interacts biochemically with the CPC, and that the spindle assembly phenotype in XSgo2-depleted extracts is similar to that caused by mild perturbation of the CPC. I appreciate that Aurora B protein levels are largely unaffected in XSgo2-depleted extracts, but the add-back experiment would definitively show that the spindle assembly phenotype is specific to XSgo2 depletion. Related to this comment, it would be useful to include a Coomassie blue-stained gel of XSgo2 immunoprecipitates from egg extracts.

2. In examining the chromosomal localizations of MCAK, CENP-A, Aurora B, Bub1, and Condensin, why are chromosomes prepared in cycled extracts for some experiments and CSF extracts (MCAK in particular, Figure 5) for others? Chromosome preparations from cycled extracts are more physiologically relevant (especially from the standpoint of kinetochore/centromere organization during mitosis), so it would be useful to include phospho-MCAK protein levels on chromosomes in cycled extracts.

3. The effect of XSgo2 depletion on MCAK localization at centromeres in spindles (Figure 4) appears to be more severe than its effect on chromosome preparations (Figure 5). However, this is difficult to gauge because quantitative data on MCAK levels at centromeres is not provided in Figure 4. Does the attachment of kinetochores to microtubules influence MCAK protein levels at centromeres in the absence of XSgo2?

4. I am unconvinced that abnormally high levels of active MCAK are present along chromosome arms in XSgo2 extracts. If immunostaining is the best way to test this, I would suggest presenting MCAK fluorescence intensities as a ratio of pS196-MCAK to total MCAK. Immunoblotting (instead of immunostaining) of chromatin preparations with MCAK and pS196-MCAK antibodies might also be a reasonable alternative.

5. The results section describing the PP2A-B56 biochemistry experiments is written strangely. In particular: "The antibody against PP2A-B56 hardly detects this protein... However a tagged version of PP2A-B56 exogenously added to ... specifically with XSgo2 and not with XSgo1." The XSgo2

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affinity-purification experiment in 7A successfully identified the B56 subunit from extracts. A similar experiment should be performed with XSgo1 to convincingly show that the two proteins interact with distinct PP2A complexes.

Minor points:
1. Throughout the manuscript, I urge the authors to use the term "Meiosis" instead of "Mitosis" when describing the cell cycle state of the extract, since all metaphase arrests were accomplished using CSF extract.

2. Error bars are needed in Figure 3B.

3. There is a typo on the top of p. 5: Although the primary signal to attract Sgo proteins ... (Kawashima et al.). Aurora B is also required..." The underlined period should be a comma.

4. Generally, the introduction is very long and hard to read. This could be due to the nature of the field, currently, but I feel that the introduction could be tightened up significantly.

1st Revision - authors' response 07 December 2011

Referee #1 (Remarks to the Author):

Shugoshins are important regulators of chromosome segregation and have two major functions attributed to them: the protection of centromere cohesion and sensing of tension between sister kinetochores. (…) Here the authors show that XSgo2 does not participate in the protection of centromeric cohesion, but that it is required for proper bipolar spindle formation and chromosome alignment. They show evidence that XSgo2 is not required for the localization of the Chromosome passenger complex at centromeres, but that it might be required for its full activation in the extracts. Because the CPC has previously been demonstrated to be required for proper spindle assembly in this system, this could offer an explanation for the spindle phenotypes of XSgo2-depleted extracts. Finally, the authors show that XSgo2 pulls down a different PP2A holoenzyme than XSgo1.

This is a very nice characterization of Xenopus Sgo2 protein. Although much has been published on Sgo proteins in general, a clear consensus has not emerged and further information from the Xenopus system is very valuable. The paper is extremely well written, gives a good overview of the background and the quality of the data is high.

The paper could however be strengthened by the following modifications:

1. The authors demonstrate nicely that XSgo2 associates with the B56e subunit-containing PP2A and propose that the specific functions of XSgo2 in spindle function are mediated through this form of PP2A. What is the effect of depletion of B56e on spindle assembly in these extracts. It would greatly strengthen the paper to show some data to support this hypothesis.

The association of XSgo1 and XSgo2 with different B56 subunits, shown in the last figure of the manuscript, led us to propose that at least some of their specific functions are mediated by these distinct interactions. Dissecting the exact contribution of B56 subunits requires a large amount of specific antibodies and other reagents to reconstitute PP2A and this is beyond our possibilities right now. Nevertheless, to get some additional data, as requested by the reviewer, we asked Satoru Mochida (now included in the author list) for antibodies specific against B56epsilon and B56gamma. Although in principle these antibodies work for depletion (see Mochida et al (2009) EMBO J 28:2777-85) we have been unable to obtain an efficient depletion of B56e. In contrast, we have found that B56g is efficiently depleted and codepletes Sgo1 from the extract, but not Sgo2. Depletion of Sgo1 does not codeplete B56g, suggesting that this form of PP2A has additional partners. Similarly, we found that depletion

of Sgo2 does not codeplete B56e. Thus, the new results further support the idea that Sgo1 and Sgo2 bind to different versions of PP2A B56 subunit. We have added these data to Fig. 7D and also made a new Supplementary Figure 6.

2. The conclusion that depletion of XSgo2 decreases Aurora B activity is based on the kinase assay shown in Figure 6C. To make this conclusion this experiment should be more rigorous. Are the cellular levels of Aurora B the same in the three conditions? The percentages of activity should be shown for three independent experiment repeats with an indication of the error.

In answer to the referee´s question, the levels of Aurora B do not change upon depletion of Sgo1 or Sgo2, as shown in Fig 1D of the original manuscript and now better quantitated in the new Supplementary Figure 5A. The signals of the phospho-INCENP (radioactivity) were normalized to the amount of immunoprecipitated INCENP, measured from the Coomassie-stained gel. The experiment presented in the first version of the manuscript had been done twice: one is the experiment shown in Figure 6C of the original manuscript and the other an experiment in which only Sgo2 depletion (not Sgo1 depletion) was tested along with a mock depletion. In both cases, the phosphorylation of the immunoprecipitated INCENP was reduced in the absence of Sgo2. To respond to the referee, we decided to repeat the experiment twice, but we did not find the same results. We then performed additional experiments. In total, we have performed eight experiments and our conclusion is that, at least as measured with this assay, Sgo2 depletion does not consistently affect the activity of the soluble CPC. We have therefore changed this conclusion in the main text (end of page 10 of the current manuscript) and prepared a new Supplementary Figure showing these results (Supplementary Figure 5D). We have also prepared a figure for the reviewer showing all the images from these experiments in the last page of this document. In any case, we still believe that Sgo2 does affect the activity of the CPC bound to chromatin, as reflected by the results of immunofluorescent stainings shown and quantitated in Figure 6D of the previous version, now Figure 5C)

3. Some experiments show error bars even though only two experiments are included. E.g. Fig. 2B and S3.

We have now increased the number of experiments in some cases (Figure 2, Figure 4, Supplementary Figure 3) and changed the type of representation in other cases (Figure 5 in the current version).

Referee #2 (Remarks to the Author):

Xenopus Sgo2 regulates the spindle assembly pathway mediated by the CPC Rivera et al.

(…) This study describes the identification of Xenopus Sgo2 and dissects the function of XSgo1 and XSgo2 in this model organism. While XSgo1 controls centromeric cohesion, this study shows that XSgo2 is not involved in the protection of centromeric cohesin but instead regulates Aurora B kinase activity, MCAK localization, spindle assembly and chromosome alignment. Moreover, the two XSgo proteins interact with different PP2A regulatory subunits proposed to dictate their non-overlapping functions. Overall this is an interesting study on an important group of proteins, but at the moment a number of the conclusions are not evidence based. Below I have listed the points that need to be improved.

1) The authors conclude that the "presence of an abnormally large fraction of active MCAK depolymerase along chromosome arms" would hinder spindle assembly in the absence of XSgo2 (p.11). The experiments shown in figure 5 do not convincingly show this, and the authors should demonstrate that the effect of Sgo2 depletion on spindle assembly is indeed dependent on MCAK. Co-depletion of MCAK and XSgo2 is expected to revert the spindle assembly defect (fig 3). This experiment should be done.

Our results show that depletion of Sgo2 alters the distribution of active MCAK on chromatin. In contrast, depletion of MCAK removes MCAK from chromatin, from the spindle and from the soluble extract, leading to the formation of huge aggregates or “sunburst arrays” with long microtubules (Walczak 1996, Ohi 2004). Thus, it cannot be expected that double depletion of Sgo2 and MCAK will rescue the spindle assembly defect observed in the absence of Sgo2. We have performed the requested experiment once and indeed, there are no bipolar spindles in the doubly depleted extracts. Nevertheless, we have realized that classification of spindle defects is rather subjective and that the appearance of spindles with low microtubule density, which we explained in the original manuscript by the “excess” of active MCAK along arms, is variable. At present it is difficult to dissect the specific routes that produce distinct defects. For example, Haspin depletion but no Sgo2 depletion affects spindle length (see new Figure 6C). We have therefore decided to lessen our statement in the text: “Thus, the balance between active and inactive MCAK along chromosome arms is changed in the absence of Sgo2 and this likely contributes to the observed defects in spindle

assembly" (page 10 in current version). In addition, we have merged in a single figure and a single paragraph all the MCAK-related results (Fig 4 and Fig 5 in the previous version are now the new Figure 4). We have also shortened in the Discussion the paragraph commenting these results.

2) The authors suggest that the decrease in Aurora B kinase activity (fig. 6) seen after XSgo2 depletion is responsible for the spindle assembly defects, but this is not formally shown. The phenotype of Sgo2 depletion is not as strong as for CPC depletion (p. 15), and in the discussion the authors provide a plausible explanation for this: Sgo2 and Haspin may act together in activating Aurora B and hence depletion of one still allows some activity by the other. This translates into an easy experiment (codepletion of Haspin and XSgo2 should enhance the functional defects shown in fig 3) that would strengthen the message of the paper.

This is a very good suggestion. We have performed the experiment requested by the reviewer and found that double depletion of Haspin and Sgo2 does enhance the spindle assembly defects. We have included these results in the main text, both in Results (page 12 and new Figure 6) and in Discussion (page 15).

3) In figure 7 it is demonstrated that XSgo1 and XSgo2 interact with different regulatory subunits of PP2A. This is potentially very interesting but unfortunately no evidence is provided that this differential interaction relates to the different functions of the two Sgo proteins, as presented in the model of figure 8. The authors should provide at least some evidence that this might be the case. B56e and B56g should be depleted and the consequence on centromeric cohesin and on Aurora B localization and activity (pT248) should be determined.

We obtained sera raised against these B56 subunits from Satoru Mochida (now included as an author) and performed some depletion experiments. Interestingly, we have found that B56gamma depletion codepletes Sgo1 and consistent with that, chromosomes assembled in B56gamma depleted extracts have little Sgo1 at centromeres whereas the amount of Sgo2 in the extracts, its centromeric targeting and recruitment of MCAK are not affected. Unfortunately, we have been unsuccessful in performing B56epsilon depletion. In any case, we have included some of these new data in the last section of results (Figure 7D and new Supp Figure 6). One important result is that depletion of Sgo1 does not perturb significantly the levels of B56gamma in the extracts, which indicates that B56gamma has additional partners and its depletion could therefore affect pathways other than those governed by

Sgo1. In consequence, one should not expect that the depletions of the B56 subunits will phenocopy the Sgo depletions.

In any case, our current data demonstrate that Sgo1 and Sgo2 have different functions and interact with distinct B56 subunits, and this is what the model in Figure 8 depicts. Whether the association of Sgo proteins with these distinct B56 subunits dictates, at least in part, their specific functions, and how this may happen, remains to be addressed. In the last sentence of the Results section we now say “Since the PP2A-B subunits are supposed to determine the substrate specificity of the phosphatase, we speculate that these specific associations contribute to the non-overlapping functions of XSgo1 and XSgo2”. We have also toned down the Discussion and removed the sentence “Our data suggest that the specific PP2A complexes associated with XSgo proteins determine the clear division of labor between XSgo1 and XSgo2” (page 17 in previous version). Finally, we have modified the last sentence of the Abstract “Our results further suggest that this functional specificity could rely on the association of XSgo1 and XSgo2 with different regulatory subunits of the PP2A complex”.

4) The finding that XSgo1 depletion results in a dramatic decrease in centromere localized Aurora B, but hardly affects centromeric T248 phosphorylation of Aurora B is puzzling and intriguing but not explained. It suggests that the very small pool of Aurora B that remains at the centromere after Sgo1 depletion is highly active and that Sgo1 may thus have an inhibitory effect on the activation of Aurora B. Since Sgo2 promotes Aurora B activation (pT248), the following scenario might be the case: While Sgo1 promotes the centromeric recruitment of Aurora B, at the same time it also inhibits its kinase activity, but this inhibitory effect is on its turn counteracted by Sgo2. If true, then it is expected that in the absence of Sgo1, Sgo2 depletion will no longer affect T248 phosphorylation of Aurora B nor the centromeric localization of MCAK. In other words, the authors should show what happens to the centromeric pT248 and MCAK levels in the XSgo1/XSgo2 double depleted cells. If it is similar to the XSgo1 single depleted cells and thus higher than in the XSgo2 single depleted cells, then this could be an explanation for how the two Sgo proteins locally control CPC activity. If the XSgo1/XSgo2 double depletion is similar to the XSgo2 single depletion, then the explanation given on p. 17 is more likely.

We found the hypothesis proposed by the reviewer very attractive and worth testing. However, we have found that double depletion of Sgo1 and Sgo2 does not rescue Aurora B-

pT248 levels or MCAK-pS196 levels at centromeres. These data are now included in Figure 5 of the current manuscript and in Supplementary Figure 4B.

5) Fig. 6A: The control antibody (RbIgG) immunoprecipitates lots of INCENP and Dasra A (lane 2), while it does not bind PPA2A-C or PP2A-B56g; (fig 7C). This hampers the conclusion that XSgo1 and XSgo2 interact with the CPC as it could be explained by a specific binding to the rabbit IgG.

We understand the reviewer´s concern. The problem is that only a very small fraction of the CPC interacts with Sgo1 and Sgo2 and we have to do long film exposures to detect it and some unspecific binding to the RbIgG becomes visible. We believe that the coIP reflects the ability of the CPC and the Sgo proteins to interact although this interaction is likely to happen on chromatin, not in the soluble extract. Consistently, when we deplete Sgo1 or Sgo2 from the soluble egg extracts, the amount of soluble CPC does not change (see new Supplementary Fig. 5A). In any case, we have replaced the western blot in Figure 6A of the original version with another blot from an experiment in which we have adjusted the amount of antibody used in each immunoprecpitation (now we also show IgGH signal) and we have also used two different Sgo2 antibodies (Figure 5A in the current manuscript). Although small in all cases, the amount of INCENP immunoprecipitated with Sgo2 or Sgo1 is larger than the amount observed with a similar amount of the control antibody.

6) Fig 6C: In contrast to figure 6D, this is not a convincing experiment to show that Sgo2 promotes Aurora B kinase activity. First, there is no evidence that the CBB stained band in the upper panel is indeed INCENP. Second, the reduction in phospho-INCENP seen in the Sgo2 depleted cells can be explained by the reduction in the amount of INCENP (i.e. the CBB band is less intense compared to the other two bands).

In answer to the referee´s concerns: (1) The band shown in CBB is INCENP because it has the expected size and is pulled down with an Aurora B antibody previously validated (first described in MacCallum 2002, MBC 13) and not with control IgG; (2) The signals of the phospho-INCENP (radioactivity) were normalized to the amount of immunoprecipitated INCENP, measured from the Coomassie-stained gel. The full gel and autorad are now shown (new Supplementary Figure 5D).

Referee 1 showed also some concerns about this experiment and requested “percentages of activity should be shown for three independent experiment repeats with an indication of the error”. As we explain in our answer to this referee, the experiment presented in the first

version of the manuscript had been done twice: one was the experiment shown in the original Figure 6C and the other was an experiment in which only Sgo2 depletion (not Sgo1 depletion) was tested along with a mock depletion. In both cases, the phosphorylation of the immunoprecipitated INCENP was reduced in the absence of Sgo2. To respond to the referee, we decided to repeat the experiment twice, but we did not find the same results. We then performed additional experiments. In total, we have performed eight experiments and our conclusion is that, at least as measured with this assay, Sgo2 depletion does not consistently affect the activity of the soluble CPC. We have therefore changed this conclusion in the main text (en of page10 of the current manuscript) and prepared a new Supplementary Figure showing these results (Supplementary Figure 5D). We have also prepared a figure for the referees showing all the images from these experiments. In any case, we still believe that Sgo2 does affect the activity of the CPC bound to chromatin, as reflected by the results of immunofluorescent stainings shown and quantitated in Figure 6D of the previous version, now Figure 5C)

7) Fig. 4B: In human cells depletion of Sgo1 chromosome alignment is severely disrupted (Salic, Cell 2004, McGuinness et al., Plos Biol 2005) due to loss of centromere cohesion. Do the authors have an explanation for the fact that this is not seen in Xenopus extracts (fig. 4)?

In human cells, reduction of Sgo1 levels by siRNA has a dramatic effect on both centromeric and arm cohesion with most chromosomes found in metaphase spreads showing completely separated sister chromatids. The lack of cohesion most likely hinders chromosome alignment. In contrast, the defect in cohesion is much more subtle in the chromosomes assembled in Sgo1 depleted extracts and affects only centromeric cohesion, not arm cohesion (see also our previous paper Rivera and Losada (2009) Chromosoma). Under these circumstances, chromosome alignment is not perturbed.

Minor points:

Fig. 1B: Please show insets with magnifications of the kinetochores , to show that XSgo1 and XSgo2 have overlapping localizations in mitosis.

p. 5: "at least vitro", should be "at least in vitro"

p. 31 legend figure 7: "Tale 1" should be "Table 1"

We have now included the insets requested and corrected the typos.

Referee #3 (Remarks to the Author):

In this interesting manuscript, Rivera et al. use frog egg extracts to study the function of Xenopus Sgo2. Surprisingly, and unlike XSgo1, XSgo2 does not regulate sister chromatid cohesion but instead influences meiotic spindle assembly. Extracts depleted of XSgo2 form monopolar structures or spindles that have reduced microtubule levels, an effect attributed to enhanced chromosome arm localization of active MCAK. XSgo2 interacts with the CPC and PP2A complexes, which leads the authors to propose that XSgo2 functions in the CPC arm of the spindle assembly pathway. XSgo2 is suggested to contribute to CPC activation, which would block MCAK activity during spindle assembly and thus promote microtubule polymerization.

Major points:

1. A major concern about the XSgo2 depletion studies is that a rescue experiment is not performed. If XSgo2 is difficult to obtain in recombinant form, is it possible to perform an add-back using the in vitro transcription/translation approach? This data seems particularly important given that XSgo2 interacts biochemically with the CPC, and that the spindle assembly phenotype in XSgo2-depleted extracts is similar to that caused by mild perturbation of the CPC. I appreciate that Aurora B protein levels are largely unaffected in XSgo2-depleted extracts, but the add-back experiment would definitively show that the spindle assembly phenotype is specific to XSgo2 depletion. Related to this comment, it would be useful to include a Coomassie blue-stained gel of XSgo2 immunoprecipitates from egg extracts.

We have attempted the rescue experiment requested by the reviewer but the in vitro translated Sgo2 (tagged or untagged) added back to a Sgo2 depleted extract could not be found at centromeres. We suspected that this could be due to codepletion of PP2A B56e so next we added both in vitro translated Sgo2 and B56e, but again we failed to observe centromere targeting. We found later that endogenous B56e is apparently not codepleted by Sgo2 depletion (see B56e blot in new Figure 7D of the revised manuscript) same as B56gamma is not codepleted in Sgo1 depletion. One possibility that the B56e remaining in the Sgo2 depleted extract is stably bound to other partners and the added back Sgo2 cannot compete for it. Even the added B56e likely fails to compete efficiently with other B56 subunits for binding to PP2A-A and PP2A-C. This is a common problem in the frog oocytes due to the

presence of stable, preassembled complexes. Thus, we may need to reconstitute and add back the whole PP2A heterotrimeric complex, but doing this with in vitro translated proteins would require diluting the extracts to a point most likely incompatible with its function, specially in terms of spindle assembly. Reconstituting the trimeric complex from bacterially expressed proteins is our next goal, but we have not succeeded yet. Another possibility is that Sgo2 is associated to a factor, other than PP2A, required for its targeting, but the mass-spec analysis of the Sgo2 affinity purified fraction does not point to a clear candidate.

If I understand correctly, the reviewer is mostly worried by the possibility that a decrease in CPC abundance (difficult to detect in the western blot presented in Fig 1D) is responsible for the spindle assembly defect. We have therefore tested more carefully the effect of Sgo1 and Sgo2 depletions on CPC levels in the extract and we conclude that there is no measurable decrease (new Supplementary Figure 5A). This is consistent with our difficulties in coimmunoprecipitating Sgo proteins and the CPC (Fig 6A in the original manuscript; 5A in the current one; see also response to referee 2, point 5). Finally, the CBB gel of Sgo2 immunoprecipitates requested by the reviewer has now been included in Supplementary Figure 1B.

2. In examining the chromosomal localizations of MCAK, CENP-A, Aurora B, Bub1, and Condensin, why are chromosomes prepared in cycled extracts for some experiments and CSF extracts (MCAK in particular, Figure 5) for others? Chromosome preparations from cycled extracts are more physiologically relevant (especially from the standpoint of kinetochore/centromere organization during mitosis), so it would be useful to include phospho-MCAK protein levels on chromosomes in cycled extracts.

The reviewer is right in that cycled extracts may be more physiologically relevant. However, we used CSF extract treated with nocodazole in Figure 5 to measure the effect of Sgo2 depletion in MCAK levels because one expert in the field, Claire Walczak recommended this condition arguing that better and more reproducible results are obtained in these extracts. Nevertheless, we have now included in the manuscript new measurements in cycled extracts to address this request (new Supplementary Figure 4).

3. The effect of XSgo2 depletion on MCAK localization at centromeres in spindles (Figure 4) appears to be more severe than its effect on chromosome preparations (Figure 5). However, this is difficult to gauge because quantitative data on MCAK levels at centromeres is not

provided in Figure 4. Does the attachment of kinetochores to microtubules influence MCAK protein levels at centromeres in the absence of XSgo2?

In order to answer the reviewer´s question we have measured MCAK levels at centromeres in cycled extracts, mock depleted or depleted of Sgo2, in the presence and absence of nocodazole. We observed no significant differences between the two conditions. We have made a Supplementary Figure to show these results (Supplementary Fig 4A).

4. I am unconvinced that abnormally high levels of active MCAK are present along chromosome arms in XSgo2 extracts. If immunostaining is the best way to test this, I would suggest presenting MCAK fluorescence intensities as a ratio of pS196-MCAK to total MCAK. Immunoblotting (instead of immunostaining) of chromatin preparations with MCAK and pS196-MCAK antibodies might also be a reasonable alternative.

We have tried immunoblotting of chromatin fractions, but we repeatedly observe a lot of MCAK in the control sample without sperm. This background prevents a useful quantitation of MCAK levels on chromatin assembled in the depleted extracts. Regarding the suggestion of representing fluorescence intensities as a ratio of pS196-MCAK to total MCAK, we have preferred not to follow it because we are not sure what that means: What we represent is the average signal of MCAK or phospho-MCAK (centromeric or from the arms) with respect to mock so the ratio between these numbers does not indicate what fraction of the total MCAK present is phosphorylated (in S196). All we can say is that there is not a similar decrease in both signals upon depletion of Sgo2, with inactive phosphoMCAK decreasing more than total MCAK. In any case, as we indicate in the response to Referee 2, point 1, we have lessened the importance of the arm bound fraction of active MCAK on the spindle defects observed in the absence of Sgo2, and now say: “Thus, the balance between active and inactive MCAK along chromosome arms is changed in the absence of Sgo2 and this likely contributes to the observed defects in spindle assembly” (page 10 in current version). In addition, we have merged in a single figure and a single paragraph all the MCAK-related results (Fig 4 and Fig 5 in the previous version are now the new Fig 4). We have also shortened in the Discussion the paragraph commenting these results.

5. The results section describing the PP2A-B56e biochemistry experiments is written strangely. In particular: "The antibody against PP2A-B56e hardly detects this protein... However a tagged version of PP2A-B56e exogenously added to ... specifically with XSgo2 and not with XSgo1." The XSgo2 affinity-purification experiment in 7A successfully identified

the B56e subunit from extracts. A similar experiment should be performed with XSgo1 to convincingly show that the two proteins interact with distinct PP2A complexes.

We have rewritten this section and also expanded it with some new results regarding B56g depletion. We would like to clarify that B56epsilon subunit poses two major difficulties for western blot analyses:

(1) its low abundance in the egg extract;

(2) its size, which coincides with that of IgG heavy chain and makes its detection in immunoprecipitates impossible even if crosslinking is employed.

We solved these problems by analyzing the fractions eluted from an affinity purification of Sgo2 with antibodies against B56 subunits (Figure 7A of the original version) and also by carrying out Sgo1 and Sgo2 immunoprecipitations from an extract containing V5-B56e (Figure 7B of the original version). In the case of Sgo1, similar analyses of an affinity purified fraction could not be performed. The antibody against Sgo1 used in this manuscript is raised against recombinant protein, not a peptide. We do have a second antibody against peptide but it its efficacy in capturing the protein from the extract is not that good and the efficiency of the antigen peptide in elution is also low. Nevertheless, B56gamma, which runs well above the IgG heavy chains, is clearly identified by western blot in Sgo1 precipitates (Fig 7C of the original version, now Figure 7A). Furthermore, our new data show that most, if not all Sgo1 in the extract is bound to this particular B56 subunit, since depletion of B56g removes Sgo1 as well (new Figure 7D).

Minor points:

1. Throughout the manuscript, I urge the authors to use the term "Meiosis" instead of "Mitosis" when describing the cell cycle state of the extract, since all metaphase arrests were accomplished using CSF extract.

Although the reviewer is right in that we are using extracts arrested in meiosis II by the CSF, there is a rather accepted consensus in the field to use the term mitosis. We would prefer not to make the change requested by the reviewer unless the editor considers it essential.

2. Error bars are needed in Figure 3B.

We have done this.

3. There is a typo on the top of p. 5: Although the primary signal to attract Sgo proteins ... (Kawashima et al.). Aurora B is also required..." The underlined period should be a comma.

This has been corrected.

4. Generally, the introduction is very long and hard to read. This could be due to the nature of the field, currently, but I feel that the introduction could be tightened up significantly.

We have shortened the Introduction in the revised version of the manuscript.

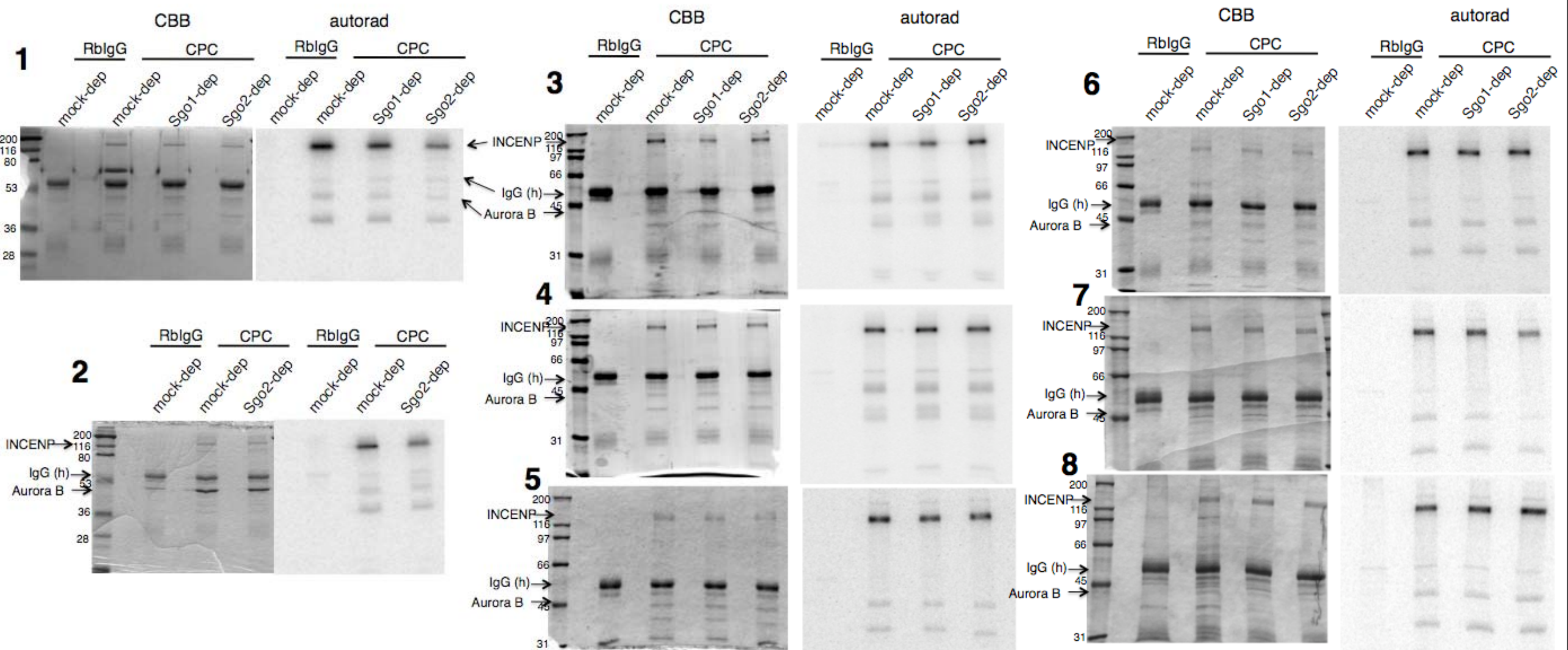

Figure for referee
1
CBB
autorad
RbIgG
CPC
mock-dep
Sgo1-dep
Sgo2-dep
200
116
80
53
36
28
2
INCENP
IgG (h)
Aurora B
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4
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97
66
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31
old experiments
new experiments

Acceptance letter 22 December 2011

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

You shall receive a formal letter of acceptance shortly!

Best wishes
Editor
The EMBO Journal

Referee #2

(Remarks to the Author)
This is a revised version of previously submitted manuscript. The authors have performed additional experiments that strengthen the message of the paper. In the point-to-point reply they also explain clearly why certain experiments requested by the referees were not feasible.
Overall, the authors have done a wonderful job in revising the manuscript and I therefore recommend it for publication in EMBO Journal.

Referee #3

(Remarks to the Author)
In the revised version of this manuscript, Rivera and colleagues have done an admirable job of addressing the reviewers' comments. Although some of my concerns still stand (namely the specificity of the Sgo2 depletion phenotype), I feel that the manuscript is publishable in its current form. Sgo2 is clearly an important protein during cell division, and the novel findings in this manuscript help to clarify its functions.

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