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Inhibitor-3 ensures bipolar mitotic spindle attachment by limiting association of SDS22 to kinetochore-bound protein phosphatase 1

Annika Eiteneuer, Jonas Seiler, Matthias Weith, Monique Beullens, Bart Lesage, Veronica Krenn, Andrea Musacchio, Mathieu Bollen and Hemmo Meyer

Corresponding author: Hemmo Meyer, Universität Duisburg-Essen

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

Editor: Hartmut Vodermaier

1st Editorial Decision 06 June 2014

Thank you again for submitting your manuscript on kinetochore PP1 regulation by SDS22 and I3 for our consideration. We have now received the comments from three expert referees (copied below), and I am pleased to inform you that all of them consider this work an important contribution warranting publication in The EMBO Journal, pending satisfactory revision of a limited number of experimental concerns and additional minor issues. In my view, the key points to address would be point 1 of referee 1 (directly assessing attachment e.g. via checkpoint protein staining) and related point 3 of referee 2 (assessing at least one Aurora B substrate as alternative, direct readout for Aurora B activity). Furthermore, the double RNAi experiment suggested by referee 2 (point 2) would provide a very important validation of the proposed model and should thus be included. Finally, any data to address the quantification- and data presentation-related points 2 (ref 1) and 1 (ref 2) would be very helpful to include in the revised manuscript.

I would therefore like to invite you to submit a revised manuscript, keeping in mind that our policy to allow only a single round of major revision makes it important to carefully answer to all points raised at this stage. 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; should you foresee a problem in meeting this three-month deadline, please let me know in advance and we could discuss the possibility of an extension.

Thank you again for the opportunity to consider this work, 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!

REFEREE COMMENTS

Referee #1:

This manuscript addresses an important questions that has been confusing in the literature until now: the function of sds22 with respect to Aurora B at kinetochores. The paper is well written and provides a clear and convincing model to resolve seemingly contradictory findings. I have only a few suggestions:

1. The discussion states that "SDS22 depletion leads to increased Aurora B activity at the kinetochore accompanied by chromosome segregation defects consistent with compromised PP1 function" (p. 11-12). Defects are expected if Aurora B activity changes in either direction (increased or decreased), and it is important to be clear about what the effect is. Increased Aurora B activity is predicted to destabilize kinetochore-microtubule attachments, which presumably leads to the chromosome misalignment shown in Figure 1. Looking at attachments directly, for example staining for cold-stable microtubules, would strengthen the argument that defects are due to increased Aurora B activity. Localization of checkpoint proteins such as Mad1 or Mad2, or other proteins that are recruited to unattached kinetochores, would be another way to test whether attachments are destabilized.

2. Measurements of Aurora B T232 phosphorylation in Figures 5 and 7 should be calculated as a ratio to total Aurora B as in Figure 2. Otherwise it is not clear whether there is a change in phosphorylation or a change in total levels of Aurora B at centromeres.

Minor points:

1. The introduction to how Aurora B localizes to centromeres by histone phosphorylation (p. 3) is incomplete. Both H3 and H2A phosphorylation are thought to contribute.

2. The text states that "whether SDS22 localizes to kinetochores was drawn into question" by the Liu et al. 2010 paper (p. 4). I don't recall that paper mentioning anything about SDS22.

3. It is not clear what "positive functions in chromosome segregation" (p. 4) means.

4. What do "controlled levels" (p. 5) mean for expression of GFP-I3? From the blot in Figure S1A, it looks like overexpression.

5. For the phospho-AurB staining in Figure 2 (and other figures), is the staining at the inner centromere or at the outer kinetochore? It's difficult to tell from the images shown. Intuitively, one would expect staining at the inner centromere because Aurora B localizes there, but other papers have shown phospho-AurB only at the outer kinetochore, which has always been confusing. It's worth pointing out if the staining here is consistent with previous results (outer kinetochore), or consistent with the inner centromere localization of Aurora B.

6. What does "warrants PP1 functionality" (p. 11) mean?

7. "manny" is a mistake on p. 12.

Referee #2:

For this paper, the authors investigated the role of Sds22 in promoting the function of the phosphataste PP1 at kinetochores. The phosphorylation dynamics at kinetochores play a central role

in ensuring the timing and fidelity of chromosome segregation. Although extensive work has focused on the kinases that regulate kinetochore function, much less is known about the counteracting phosphatases. Previous work implicated Sds22 in acting together with PP1 at kinetochores, but the nature of its role was unclear. This is in part related to disagreements in the literature about whether Sds22 localizes to kinetochores. This paper cleanly and clearly resolves this debate to provide a coherent picture of the role of Sds22 in promoting the mitotic functions of PP1. The level and quality of the experimentation in this paper is very good, and the data and observations they generate are very interesting. As such, this paper provides an important contribution and is a great fit for EMBOJ. However, there are several experiments that I think would further strengthen this paper and the author's conclusions.

1. It would be helpful if the authors could quantify the amount of GFP-PP1 at kinetochores for their experiment in Figure 2D. I realize that this might be complicated depending on the nature of the cell line, but it would be very useful to have some more precise information on the level of PP1 that localizes to kinetochores in each condition.

2. The observed change in localization for Sds22 when I3 is depleted is striking, and quite cool. As such, the authors interpret the I3 depletion phenotypes as related to the excess Sds22 at kinetochores. It would be very useful to explicitly test this model by depleting Sds22 in their I3 RNAi conditions (double RNAi). The prediction of this, at least as I understand the model, is that this should relieve the phenotype that they detect.

3. A significant component of their conclusions are related to the nature of the balance between Aurora B kinase and PP1 phosphatase for controlling the phosphorylation of kinetochore substrates. Their work is consistent with their models. However, it would be particularly useful to monitor phosphorylation downstream of Aurora B in as many ways as possible. They primarily use phospho-Aurora B staining, and I think that this is an imperfect proxy for many of the substrates that they wish to make conclusions about. There are additional phospho-antibodies against defined Aurora B substrates for which there are commercially available antibodies, or published antibodies that should be available to these authors. It would be very important for the authors to test at least one other (if not more) substrates in their various conditions.

Referee #3:

This manuscript explores the role of Sds22 and I3, two protein phosphatase 1 (PP1)-interacting proteins, in the regulation of PP1 activity during mitosis in human cells and in particular in regulating the activity of kinetochore-localised Aurora B kinase. The work resolves conflicting reports in the literature regarding kinetochore localization of Sds22 and provides a stronger rationale for understanding the positive and negative roles in PP1 function that have been ascribed to Sds22, by showing that I3 prevents association of Sds22•PP1 with the kinetochore, thereby supporting a chaperone-like function for Sds22/I3. The data showing that I3 controls the association of Sds22•PP1 with the kinetochore and that this has important consequences for Aurora B activity are novel and appear convincing based on the presented data. That the effect of Sds22 on PP1 function is complex and to an extent paradoxical has been proposed before, but here the authors provide evidence for a mechanism that can explain this. The work is discussed comprehensively in the context of earlier studies and makes a novel contribution to the field that should be of reasonably wide interest because it impinges both on the chromosome biology and protein phosphatase fields.

The following points should be addressed by the authors:

1) p.5: the rationale for using H2B-RFP and IBB-GFP to establish nuclear envelope breakdown should either be referenced or preferably described briefly in the legend to Fig. 1C and/or Fig. S1

2) p.9/Fig. 5A: it is not difficult to exclude some kinetochore localization of the mCherry signal in the the two mutant mCherry-Sds22-expressing strains due to the high uniform fluorescence level?

3) p.12 middle paragraph. The authors are correct to conclude that differences in the expression level of tagged Sds22 most likely account for the differences in whether Sds22 is seen at the kinetochores in different studies. However, in the Posch et al. (2010) study there were apparently no adverse consequences of kinetochore localization of overexpressed Sds22 whereas in the current study, whether brought about by I3 depletion or Sds22 overexpression, kinetochore localization of Sds22 seems to inhibit PP1 and cause mitotic defects due to hyperactivation of Aurora B. Is there an explanation for this discrepancy with the earlier work?

4) Merged images in several Figures - no key to the colours used in any of the merged images in either the main paper or supplementary information is given. While it is reasonably straightforward to interpret the two-colour images in most cases, it would be helpful to indicate a key to the colours in the figure legends where three channels are being merged. In Fig. S6 it is also not clear what is being merged - is it just PP1 and DAPI or mCherry as well? In general, the legends referring to the merged imaging data could be made clearer.

5) Fig. 4 - Panel D should show the significance level of the difference between siI3+siLuc and siI3+siKNL1. It might also help to add "exposure" after "short" and "long" to make it instantly clear what is meant.

6) Figure S1A - it would be helpful to show the significance of the comparison between siLuc + DOX and siI3 + DOX because if expression of GFP-I3 (resist) is suppressing the effect of I3 depletion then that comparison is expected to show a non-significant difference. That would seem to be as important as comparing control siLuc without GFP-I3 (resist) expression with siI3 + GFP-I3 (resist) expression.

7) Fig. S2 needs a scale bar - this is absent from the figure although referred to in the legend.

8) Legends to Figures S3 and S4. The labels of these two Legends are reversed and hence also in the wrong order - What is stated as "Figure S4" is actually referring to Fig. S3 and vice versa. The legend to S3A should also say "resistant"

9) Fig. S6 - was the mutant GFP-I3 used in S6C overexpressed at the same level as the wild-type version? Also, in S6B, it is not clear why data for mCherry-SDS22 expression are shown (possibly to avoid presenting the blot in two pieces?). S6B shows that each protein is expressed but doesn't tell us anything about the level of overexpression - that would need anti-NIPP1 and anti-I3 Western blot data so that fusion protein versus native protein levels could be compared. While perhaps not essential given that overexpression of each protein has an effect on PP1 localisation, such data would be helpful if available.

Some other very minor points:

p.3 paragraph 3 line 2 "implied" should be "implicated"
p.10 line 10 "or" should be "and"
p.11 line 25 "paradox" should be "paradoxical", and the next line should read "Our data are consistent . . ."
p.13 line 2 should say ". . . are best explained . . .", line 11 "By analogy, . . ." line 19 ". . . an RVXF-motif . . .", line 29 can delete "Moreover and . . ."
p.14 lines 1-2 ". . . as I3 depletion leads to increased association of SDS22 with PP1 when the latter is bound to a soluble form of KNL1." would be a better summary of the data.
p.15 last line of Discussion - ". . . removal of Aurora B from centromeres rather than a role specifically during anaphase." might read better here.
p.28 line 22 insert "the" before "indicated"
Fig. S3B Y-axis label - should say "detectable"

1st Revision - authors' response 28 August 2014

Response to reviewers

We would like to thank the referees for the generally positive assessment and helpful suggestions. We hope we have addressed the remaining points in our revised version. In particular, we have gathered additional evidence that the increase in Aurora B activity in response to manipulations of the PP1 subunits is functionally relevant. We now show that SDS22 and I3 depletion leads to increased phosphorylation of the Aurora B substrate DSN1. Moreover, we show that SDS22 and I3 depletion, or SDS22 overexpression, specifically increases BubRI localization to kinetochores as evidence for a weakened spindle attachment and consistent with higher rather than lower Aurora B activity. Secondly, we provide evidence that siRNA-mediated reduction of SDS22 levels can partially rescue the effect of I3 depletion, which is consistent with our model that the inhibitory effect of I3 depletion is due to failure to prevent SDS22 targeting to kinetochore-associated PP1. Please see below for a more detailed response also to the other comments.

Referee #1:

"This manuscript addresses an important questions that has been confusing in the literature until now: the function of sds22 with respect to Aurora B at kinetochores. The paper is well written and provides a clear and convincing model to resolve seemingly contradictory findings. I have only a few suggestions:

1. The discussion states that "SDS22 depletion leads to increased Aurora B activity at the kinetochore accompanied by chromosome segregation defects consistent with compromised PP1 function" (p. 11-12). Defects are expected if Aurora B activity changes in either direction (increased or decreased), and it is important to be clear about what the effect is. Increased Aurora B activity is predicted to destabilize kinetochore-microtubule attachments, which presumably leads to the chromosome misalignment shown in Figure 1. Looking at attachments directly, for example staining for cold-stable microtubules, would strengthen the argument that defects are due to increased Aurora B activity. Localization of checkpoint proteins such as Mad1 or Mad2, or other proteins that are recruited to unattached kinetochores, would be another way to test whether attachments are destabilized."

To address this point we analyzed recruitment of the checkpoint protein BubRI. As expected, we find a specific increase of BubRI, which is indicative for destabilized kinetochore-microtubule attachments and activation of the SAC. This is true in the case of SDS22 or I3 depletion (new Fig. 2F), but also for SDS22 overexpression (new Fig. 6E), which clearly supports our model that too little or excess SDS22 affects PP1. Together with the already shown increase in AurB auto-phosphorylation (Fig. 2A) and our new data on increased phosphorylation of the AurB substrate Dsn1 (new Fig. 2D,E), it also further clarifies that it is the increase (rather than loss) in AurB activity that causes the segregation errors.

"2. Measurements of Aurora B T232 phosphorylation in Figures 5 and 7 should be calculated as a ratio to total Aurora B as in Figure 2. Otherwise it is not clear whether there is a change in phosphorylation or a change in total levels of Aurora B at centromeres."

The reason why we had not included the ratios in the overexpression experiments was technical issues regarding the triple labeling (mCherry-SDS22, AurB, pAurB). We have now established the triple color detection and quantification, and included the new data accordingly as requested (new Fig. 6). Consistently, we observed a significant and specific increase also in the ratio of pAurB/AurB upon SDS22 wildtype overexpression.
As for the anaphase experiments (now Fig. 8), the fact that AurB is absent in control

cells precludes the calculation of ratios. In fact, the point that we make for anaphase is that AurB persists (which active) compared to full removal in control cells, as opposed to the situation in metaphase where we find that the correctly localized AurB is more active than in control cells.

Minor points:

1. The introduction to how Aurora B localizes to centromeres by histone phosphorylation (p. 3) is incomplete. Both H3 and H2A phosphorylation are thought to contribute.

Thank you for pointing out this mistake. We now also refer to the H2A phosphorylation.

"2. The text states that "whether SDS22 localizes to kinetochores was drawn into question" by the Liu et al. 2010 paper (p. 4). I don't recall that paper mentioning anything about SDS22."

This is probably because Liu and colleagues used the alternative nomenclature. In the first paragraph of their results, they state that they failed to detect PPP1R7 (which is SDS22) or PPP1R11 (which is I3) at kinetochores. As this was 'data not shown' and contrasted with results from Posch and colleagues, we redid these experiments as shown in the manuscript.

3. It is not clear what "positive functions in chromosome segregation" (p. 4) means.

To be clearer, we now state that "…both factors are required for faithful chromosome segregation…"

"4. What do "controlled levels" (p. 5) mean for expression of GFP-I3? From the blot in Figure S1A, it looks like overexpression."

We removed this for clarity. The expression levels on the blot speak for themselves.

"5. For the phospho-AurB staining in Figure 2 (and other figures), is the staining at the inner centromere or at the outer kinetochore? It's difficult to tell from the images shown. Intuitively, one would expect staining at the inner centromere because Aurora B localizes there, but other papers have shown phospho-AurB only at the outer kinetochore, which has always been confusing. It's worth pointing out if the staining here is consistent with previous results (outer kinetochore), or consistent with the inner centromere localization of Aurora B."

We are not confident to discriminate between both possibilities based on our data and can therefore not contribute to the discussion at that point. Crucially, we show that SDS22 and I3 are critical to balance the population of Aurora B at kinetochores and that this is functionally relevant for chromosome segregation.

"6. What does "warrants PP1 functionality" (p. 11) mean?"

We changed this to "ensures PP1 activity".

"7. "manny" is a mistake on p. 12."

This has been corrected.

Referee #2:

"For this paper, the authors investigated the role of Sds22 in promoting the function of the phosphataste PP1 at kinetochores. The phosphorylation dynamics at kinetochores play a central role in ensuring the timing and fidelity of chromosome segregation. Although extensive work has focused on the kinases that regulate kinetochore function, much less is known about the counteracting phosphatases. Previous work implicated Sds22 in acting

together with PP1 at kinetochores, but the nature of its role was unclear. This is in part related to disagreements in the literature about whether Sds22 localizes to kinetochores. This paper cleanly and clearly resolves this debate to provide a coherent picture of the role of Sds22 in promoting the mitotic functions of PP1. The level and quality of the experimentation in this paper is very good, and the data and observations they generate are very interesting. As such, this paper provides an important contribution and is a great fit for EMBOJ. However, there are several experiments that I think would further strengthen this paper and the author's conclusions."

"1. It would be helpful if the authors could quantify the amount of GFP-PP1 at kinetochores for their experiment in Figure 2D. I realize that this might be complicated depending on the nature of the cell line, but it would be very useful to have some more precise information on the level of PP1 that localizes to kinetochores in each condition."

We performed the requested quantifications and confirmed that there was no significant difference in the various conditions. The data is now included in the new Fig. 3.

"2. The observed change in localization for Sds22 when I3 is depleted is striking, and quite cool. As such, the authors interpret the I3 depletion phenotypes as related to the excess Sds22 at kinetochores. It would be very useful to explicitly test this model by depleting Sds22 in their I3 RNAi conditions (double RNAi). The prediction of this, at least as I understand the model, is that this should relieve the phenotype that they detect."

This is in fact a difficult experiment. The model indeed says that excess SDS22 binding to PP1 is the basis for the inhibitory effect of I3 depletion, and therefore codepletion of SDS22 should relieve this inhibition. However, SDS22 is also essential to activate PP1 and therefore SDS22 depletion will reduce PP1 activity (as we show in the manuscript). Nevertheless, after extensive testing we have found conditions where moderate codepletion of SDS22 (24 hours compared to 48 h depletion of I3) leads to a partial but significant and reproducible rescue of the effect of I3 depletion on Aurora B autophosphorylation (now Fig. E6). We agree with this referee that this result strongly supports our model, albeit the degree of rescue is expectedly low.

"3. A significant component of their conclusions are related to the nature of the balance between Aurora B kinase and PP1 phosphatase for controlling the phosphorylation of kinetochore substrates. Their work is consistent with their models. However, it would be particularly useful to monitor phosphorylation downstream of Aurora B in as many ways as possible. They primarily use phospho-Aurora B staining, and I think that this is an imperfect proxy for many of the substrates that they wish to make conclusions about. There are additional phospho-antibodies against defined Aurora B substrates for which there are commercially available antibodies, or published antibodies that should be available to these authors. It would be very important for the authors to test at least one other (if not more) substrates in their various conditions."

We fully agree. In the meantime, we got hold of a phospho-specific antibody to the Aurora B substrate protein DSN1. Consistent with our model, we measured a specific increase in DSN1 phosphorylation upon SDS22 or I3 depletion, thus proving that the increase in AurB activity is functionally relevant. The data is now included in Fig. 2D.

Referee #3:

"This manuscript explores the role of Sds22 and I3, two protein phosphatase 1 (PP1)-interacting proteins, in the regulation of PP1 activity during mitosis in human cells and in particular in regulating the activity of kinetochore-localised Aurora B kinase. The work resolves conflicting reports in the literature regarding kinetochore localization of Sds22 and provides a stronger rationale for understanding the positive and negative roles in PP1 function that have been ascribed to Sds22, by showing that I3 prevents association of Sds22•PP1 with the kinetochore, thereby supporting a chaperone-like function for Sds22/I3. The data showing that I3 controls the association of Sds22•PP1 with the kinetochore and that

this has important consequences for Aurora B activity are novel and appear convincing based on the presented data. That the effect of Sds22 on PP1 function is complex and to an extent paradoxical has been proposed before, but here the authors provide evidence for a mechanism that can explain this. The work is discussed comprehensively in the context of earlier studies and makes a novel contribution to the field that should be of reasonably wide interest because it impinges both on the chromosome biology and protein phosphatase fields. *The following points should be addressed by the authors:*

1) p.5: the rationale for using H2B-RFP and IBB-GFP to establish nuclear envelope breakdown should either be referenced or preferably described briefly in the legend to Fig. 1C and/or Fig. S1"

We have now done so in the legend to Fig. 1.

"2) p.9/Fig. 5A: it is not difficult to exclude some kinetochore localization of the mCherry signal in the the two mutant mCherry-Sds22-expressing strains due to the high uniform fluorescence level?"

In confocal sections, the signal at kinetochores is indeed easily visible in SDS22 wildtype overexpressing cells, and clearly absent in the case of SDS22 mutant proteins that cannot bind PP1 (now Fig. 6A). The results are supported functionally by the specific effect on Aurora B phosphorylation (now Fig. 6C) and, in the new Fig. 6E, also on BubRI recruitment.

"3) p.12 middle paragraph. The authors are correct to conclude that differences in the expression level of tagged Sds22 most likely account for the differences in whether Sds22 is seen at the kinetochores in different studies. However, in the Posch et al. (2010) study there were apparently no adverse consequences of kinetochore localization of overexpressed Sds22 whereas in the current study, whether brought about by I3 depletion or Sds22 overexpression, kinetochore localization of Sds22 seems to inhibit PP1 and cause mitotic defects due to hyperactivation of Aurora B. Is there an explanation for this discrepancy with the earlier work?"

In the study of Posch and colleagues, SDS22 overexpression was merely used to determine the localization of SDS22, while the functional studied were performed in SDS22 knockdown cells. Thus, to our knowledge, the effects of SDS22 overexpression were not studied in detail. In any case, our in vitro and in vivo observations are consistent with (and explain) the negative effects of SDS22 overexpression on PP1 activity and Aurora/Ipl1 regulation in yeast.

"4) Merged images in several Figures - no key to the colors used in any of the merged images in either the main paper or supplementary information is given. While it is reasonably straightforward to interpret the two-color images in most cases, it would be helpful to indicate a key to the colors in the figure legends where three channels are being merged. In Fig. S6 it is also not clear what is being merged - is it just PP1 and DAPI or mCherry as well? In general, the legends referring to the merged imaging data could be made clearer."

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also in the wrong order - What is stated as "Figure S4" is actually referring to Fig. S3 and vice versa. The legend to S3A should also say "resistant".”

Many thanks for the detailed and helpful suggestions. The points 4-8 have all been corrected in the revised manuscript.

“9) Fig. S6 - was the mutant GFP-I3 used in S6C overexpressed at the same level as the wildtype version? Also, in S6B, it is not clear why data for mCherry-SDS22 expression are shown (possibly to avoid presenting the blot in two pieces?). S6B shows that each protein is expressed but doesn't tell us anything about the level of overexpression - that would need anti-NIPP1 and anti-I3 Western blot data so that fusion protein versus native protein levels could be compared. While perhaps not essential given that overexpression of each protein has an effect on PP1 localisation, such data would be helpful if available.”

We have fully addressed these concerns. First, we have confirmed equal expression of wild-type and mutant GFP-I3 (now expanded view Fig. E7F). Second, we have rerun the Western blots of relevant lysates to avoid cutting the blot in two pieces (formerly Fig. S6B, now E7B). Third, we have rerun the lysates of overexpressing cells to probe for the endogenous/exogenous protein ratios (now expanded view Fig. E7C)

“Some other very minor points:
p.3 paragraph 3 line 2 "implied" should be "implicated"
p.10 line 10 "or" should be "and"
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p.28 line 22 insert "the" before "indicated"
Fig. S3B Y-axis label - should say "detectable"

All these minor errors have been corrected in the revised manuscript. Many thanks for pointing them out.

2nd Editorial Decision 03 September 2014

Thank you for submitting your revised manuscript on Mre11 DNA recognition for our consideration. It has now been assessed once more by one of the original referees (see comments below), who raises no further reservations. I am therefore happy to inform you that we now consider this work publishable in The EMBO Journal, pending addressing of a few remaining editorial concerns:

- to make the title more widely accessible, I would propose altering it to the somewhat more explicit phrasing "Inhibitor-3 ensures bipolar mitotic spindle attachment by limiting association of SDS22 to kinetochore-bound protein phosphatase 1"

Referee #2:

For this revised paper, the authors have successfully addressed each of my comments and suggestions from my previous reviews. The combination of these changes have further strengthened

what is an interesting and important paper. As such, I find this paper suitable for publication in EMBO Journal and congratulate the authors on their excellent work.

