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PICH and BLM Limit Histone Association with Anaphase Centromeric DNA Threads and Promote Their Resolution

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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 31 January 2011

Thank you again for submitting your manuscript for our consideration. We have now received the comments of three expert reviewers on it, which you will find copied below. Both the referees and the editors agree that the proposes new role for a PICH-BLM complex in nucleosome remodeling of anaphase bridges is of interest and potential importance. We should therefore be happy to invite a revised version of the study for further consideration, nevertheless there are a number of substantive concerns raised in all three reports that indicate that the main interpretations are not yet fully warranted based on the presented data, and that additional evidence will be required to decisively support the conclusions.

While I would like you to respond to all the points raised here in your revision, I do feel that some of the major points can be considered further-reaching and not directly required to support the main novel messages (e.g. ref 1 pt 2, ref 2 pt 5). There are however also a number of key points, addressing of which will be a prerequisite for obtaining full referee support and warranting publication:
- to better control the in vivo experiments to strongly support that nucleosome depletion of anaphase threads indeed depends on PICH/BLM (see ref 1 pt 7, ref 2 pt 2, ref 3pt 5)
- to strengthen the still somewhat preliminary in vitro nucleosome remodeling assays, including stronger evidence to rule out alternative functions as helicase or translocase (see ref 1 pt 3, ref 2 pt 3)
- to attempt a PICH knockdown rescue with wild-type vs the K128A ATPase mutant (see referee 1 pt 4)
In case you that you should be able to address these main points to the referees' satisfaction, then we should in principle be happy to ultimately offer publication of the study.

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In addition to these main issues, it is clear that the caveat raised by referee 3 pt 6 concerning potential discrepancies with published work on anaphase threads in PICH/BLM-depleted cells will at least need to be discussed. Concerning the inclusion of negative data, I agree with referee 3 that the data excluding an interphase function of PICH in preventing SCEs are important and should not be relegated to the supplementary material, although you may want to consider deemphasizing this part in the text (as suggested by referee 1). On the other hand, the inclusion of the negative data on PICH in the spindle checkpoint has been criticized by all three reviewers - and I have to agree. I realize that you have probably spent considerable efforts in trying to understand the initially claimed checkpoint role of PICH, and later to convincingly correct this notion, and thus I do sympathize with you wishing to have these important negative data included here. Nevertheless given that Hubner et al reported essentially the same correcting results already more than one year ago, I strongly feel that dedicating the first two paragraphs to this problem, and to results only shown in the supplementary section, starts the paper on a very weak note. Maybe these paragraphs could also be moved to the supplement as well, with only a brief sentence (possibly in the SCE section) such as "Consistent with the findings of Hubner et al 2010, we independently verified that PICH is not required for the spindle checkpoint (see suppl...)" to reference it?

When preparing your revision, please remember that it is EMBO Journal policy to allow a single round of major revision only, and that it will thus be important to diligently answer to all the various major and minor points raised at this stage. Please also bear in mind that your letter of response will form part of the Peer Review Process File, and will therefore be available online to the community in the case of publication (for more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html). Finally, please make sure to include brief 'Author Contribution' section in the final text. In any case, please do not hesitate to get back to us should you need feedback on any specific issue regarding this decision or your revision.

With best regards,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

In this manuscript, Yu and colleagues isolated a new complex containing PICH and BLM (the helicase mutated in Bloom syndrome).They demonstrated that PICH has ATP-dependent nucleosome remodeling activity in vitro, and is required for BLM localization to anaphase centomeric threads. Moreover, they showed that PICH and BLM-depleted cells contain ahaphase threads with centromere and histone markers. They proposed a model that that PICH and BLM unravel centromeric chromatin and keep anaphase DNA threads mostly free of histones, thus allowing a spatiothemporal window for their resolution by DNA repair enzymes.
Most of the data in the manuscript are of high quality. The newly identified PICH-BLM complex and its proposed mechanism of action are novel and should be of broad interest to the DNA repair and chromatin remodeling field. However, there are a few issues with this manuscript that need to be addressed before I can recommend its publication in EMBO.
1. This manuscript should be better organized. Some text and figures are not central to the main theme of the paper (For example, Figure S1 and the part that PICH is not required for spindle checkpoint). They should be either removed or shortened to a paragraph in the Discussion. The negative data of SCE can also be transferred to Supplementary figures, and its relevant text can be shortened. The cellular localization data of BLM ad PICH may also be transferred to Supplementary figures.
2. The authors should focus on their newly identified PICH-BLM complex and demonstrate that this complex is required for its proposed roles. So far, the data on this complex is quite superficial. The data in Figure S3, which mapped a potential BLM-interaction domain to the C-terminus of PICH, should be in the regular figures. In addition, the authors should expand this type of analyses by using point mutants. The idea is that they may be able to identify point mutants that disrupt interactions

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between PICH and BLM, and then examine whether these point mutants inactivate the activity of PICH in vivo, such as suppression of micronuclei formation and localization of BLM to anaphase threads. If they do, the data would suggest that formation of the PICH-BLM complex is required for normal chromosome segregation.
3. The nuclesome remodeling activity of PICH is quite weak compared to CHD1 and RSC. Does PICH have nucleosome-stimulated ATPase activity? Can authors try other nucleosome remodeling assays? The bona fide SNF2 remodeling enzymes often contain other domains that interact with histones, such as CHD or bromo domains. Does PICH have any such motifs? If not, the authors may want to investigate the possibility whether PICH is a DNA helicase or translocase.
4. The authors should investigate whether the ATPase point mutant of PICH (K128A) can rescue other phenotypes of siPICH cells, such as BLM localization to anaphase threads, and absence of histone H2B on threads, etc. This will reveal whether the remodeling activity of PICH is required for loading of BLM to the bridges.
5. In Figure 1B, the Western blotting of PICH showed high background in the 6 lanes on the right. The major bands do not appear to have the same mobility as those on the left, and it is not clear to me which band is PICH.
6. Figure 3D needs mock-treated cells as controls.
7. The authors claimed (Page 13, 2nd paragraph) that in BLM-RNAi cells, about 44% PICH-positive threads contained histones. What is the percentage in mock-treated cells? Can authors verify this result using BLM patient-derived cell line and the same line complemented by exogenous BLM?
8. In Figure 15, the authors interpreted their findings that PICH and CHD1 moved nucleosomes toward the central positions on DNA. They stated that the result is consistent with the fact that the helicase domains of both PICH and Chd1 belong to the SNF2 family of helicases. This statement is somewhat midleading because RSC is also a SNF2 helicase, even though it mobilizes nucleosomes to the border positions.

Referee #2 (Remarks to the Author):

Sister chromatid cohesion is mediated by DNA catenation and the cohesin complex. The recent discovery of DNA threads in anaphase cells raises questions as to the role of DNA catenation at centromeres. So far, the helicase proteins PICH and BLM have been shown to be localized at the DNA threads where they are suggested to be involved in anaphase resolution. Kei, et al. now report that PICH recruits BLM to the DNA threads through protein-protein interaction. Interestingly, they show that PICH carries a nucleosome remodeling activity, suggesting that PICH may remove the nucleosome to allow the expansion of the threads between separating centromeres.

Considering the related papers on this topic (Chan et al. EMBO J 2007, Wang et al. J Cell Sci 2010), I think that the novel point of this manuscript is the interaction between PICH and BLM. The authors try to address the relationship of these proteins and their molecular functions in resolving the DNA threads; however, no particular relationship between these proteins, other than localization, is shown. Moreover, the results presented in this manuscript do not sufficiently support the authors' main proposal that 'centromeric DNA threads are resolved by limiting histone incorporation'.

Comments:
1. The first paragraph of the results is a repetition of recently published results (Hubner et al. Chromosoma, 2010), so that this could be removed or dramatically shortened.

2. Although the authors suggest that histone remains at the DNA threads in PICH RNAi cells, a control is lacking in this experiment. Do the authors mean that the ratio of the amount of histone per DNA thread is increased by PICH RNAi? If so, quantitative data with full controls should be presented. The presented data may represent simply that the number of threads is increased in PICH or BLM RNAi cells.

3. It is interesting that the PICH K128A mutant loses the nucleosome sliding ability in vitro as well as the ability to resolve DNA threads in vivo. However, since this in vitro activity of PICH is marginal and the in vivo activity is not supported (see comment 2), it is premature to conclude that PICH acts as a chromatin remodeling enzyme to resolve DNA threads. I would like to see the canonical helicase assay rather than the nucleosome sliding assay.

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4. Considering that both PICH and BLM helicases are required to resolve DNA threads at centromeres, it would be nice to analyze these proteins in parallel in Fig. 6B and 6D.

5. IP products of PICH should be tested in vitro by mixing with BLM (Fig. 6D). Similarly, BLM IP could be analyzed by mixing with PICH. Usage of mutant proteins will further strengthen the conclusion.

6. In Fig 6B, I wonder whether BLM localizes at the threads in siPICH+GFP-PICH-K128A cells.

Referee #3 (Remarks to the Author):

1. p4 Introduction "Because RNAi-depletion or chemical inhibition of topoisomerase..." should be amended to "Because depletion or chemical inhibition of topoisomerase..." since in the papers cited various methods (RNAi, but also genetic modification through gene targeting) are used to deplete the protein.

2. This manuscript reports that PICH is not required for the spindle checkpoint. This finding is consistent with the report by Hubner et al 2010 and attention should be drawn to this earlier publication not only in the introduction, but also in the relevant results section (p. 6).

3. The manuscript goes onto to report the binding of PICH and BLM, including expression of the recombinant proteins in insect cells. This association appears to involve hyperphosphorylated PICH and while detectable in G1/S, is stronger in M phase. Truncated forms of PICH suggest that the BLM-interacting domain resides in the C-terminal region, although contribution of the N-terminal region to this interaction could not be fully investigated due to failure of this domain to express well in HeLa cells. While PICH can be localised both in the cytoplasm and nucleus, BLM is largely restricted to the nucleus and nucleolus and shows poor colocalisation with PICH in interphase cells. They go onto to show that, unlike BLM deficiency, depletion of PICH has no effect on SCE levels. They conclude that PICH is not required for BLM function in interphase. This aspect of the study is interesting and novel.

4. Consistent with earlier reports (Baumann, 2007; Chan, 2007) they show that in anaphase cells PICH localises to DNA threads that are also coated with BLM. Some of these threads are ultrafine, being undetectable by DAPI staining and lacking histones. They confirm that the localisation of BLM to these threads is PICH-dependent, which is consistent with Chan et al 2007, who reported that BLM associates with PICH-coated anaphase threads at a later stage. They also confirm that many (although not all) of the ultrafine threads connect centromeres. In this m/s they go on to show that depletion of PICH, like depletion of BLM, increases the incidence of micronuclei, many of which are CREST-positive. Moreover, live imaging of cells expressing H2B:mCherry has also revealed that depletion of either PICH or BLM produces chromatin anaphase bridges.

5. Using HeLa cells expressing H2B-GFP the authors report that they see an increase in the number of anaphase cells that have PICH-associated chromatin bridges in BLM-deficient cells compared with BLM normal HeLa cells. The implication, from the data in Figures 4 and 5, is that in HeLa ~50% of anaphases have PICH threads, and only a small proportion (~1/6th) of these anaphase cells have chromatin-associated PICH fibres. In BLM-depleted HeLa ~48% of anaphases have PICH-coated connections (similar to BLM-normal HeLa cells), but of these about half have chromatin-associated fibres. The authors state that there is no increase in the number of PICH threads per anaphase cell, which implies that the numbers of PICH-associated anaphase connections that lack detectable H2B decrease in BLM-depleted cells compared to BLM-normal HeLa anaphases (the difference being replaced by histone-associated fibres). They state that in BLM-depleted cells about half of PICH fibres have H2B.

I would like to see this data presented more fully and with greater clarity (with inclusion of data on numbers of PICH-positive threads containing histones for the "normal" HeLa cells, and data on the actual numbers of connections per anaphase cell under the various conditions, and the numbers in early compared with late anaphases).

6. The authors state depletion of BLM from HeLa cells does not have any effect on the either the

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percentage of anaphase cells that contain PICH threads, or the average number of PICH threads per cells. This contrasts sharply with the findings of Chan et al. 2007 who reported, in BLM-negative cells, a significantly increased frequency of DAPI-negative PICH DNA threads (both the average number per anaphase cell, and the total number of PICH thread-positive anaphase cells). Chan et al. argued that this suggested that BLM may play a role in suppressing the accumulation of DAPI-negative PICH DNA threads in anaphase.

No reference to this difference in findings is made in the current manuscript. How do the authors account for this crucial difference? According to this earlier study, in the absence of BLM, the numbers of ultrafine, DAPI-negative PICH-associated DNA threads actually increased. This would be counter to the model proposed in this manuscript, which has the PICH-BLM complex involved in removing/preventing histone incorporation along these connecting threads.

7. Finally the manuscript investigates the nucleosome remodeling activity of PICH. The K125A mutant disrupts its activity, consistent with the earlier work of Baumann et al. 2007. BLM was found not to have any effect on this in vitro assay. These findings, on PICH's influence on nucleosome positioning, are interesting and novel.

Minor points:
8. Figure S2 - labelling error in (B).
9. Lack of scale bars for most microscopy images.
10. The title over-interprets the data presented. Evidence for PICH limiting histone incorporation is preliminary. PICH and BLM may promote resolution of anaphase centromeric DNA threads by limiting their association with histones.
11. In the Discussion (p.19) the authors state that "Unexpectedly, PICH/BLM-RNAi cells contain thin histone/CENP-A-positive chromatin threads ..." . In the data presented no CENP-A-specific antibodies have been used, only CREST antisera. Therefore the authors cannot assume that these fibres contain CENP-A.

1st Revision - authors' response 29 May 2011

Referee #1:

1. This manuscript should be better organized. Some text and figures are not central to the main theme of the paper (For example, Figure S1 and the part that PICH is not required for spindle checkpoint). They should be either removed or shortened to a paragraph in the Discussion. The negative data of SCE can also be transferred to Supplementary figures, and its relevant text can be shortened. The cellular localization data of BLM ad PICH may also be transferred to Supplementary figures.

Response: We thank the referee for these good suggestions. As suggested, we have removed the data showing that PICH is not required spindle checkpoint. We have moved the interphase localization data on BLM and PICH to supplementary figures and shorted the description of such data. On the other hand, we have elected to keep the SCE data as a main figure, based on the comments from reviewer 3 and the editor.

2. The authors should focus on their newly identified PICH-BLM complex and demonstrate that this complex is required for its proposed roles. So far, the data on this complex is quite superficial. The data in Figure S3, which mapped a potential BLM-interaction domain to the C-terminus of PICH, should be in the regular figures. In addition, the authors should expand this type of analyses by using point mutants. The idea is that they may be able to identify point mutants that disrupt interactions between PICH and BLM, and then examine whether these point mutants inactivate the activity of PICH in vivo, such as suppression of micronuclei formation and localization of BLM to anaphase threads. If they do, the data would suggest that formation of the PICH-BLM complex is required for normal chromosome segregation.

Response: As suggested by the reviewer, we have now included the PICH-BLM binding data in Figure 2. We agree with the reviewer that point mutants of PICH deficient for BLM binding (but

still capable of localizing to anaphase threads) would be extremely useful. On the other hand, we hope that the reviewer will appreciate the difficulty of obtaining such a separation-of-function mutant without the guidance of structural information. Indeed, we attempted to make such mutants, but failed. In particular, we made tens of small deletion mutants (each with 5 residues deleted) in the C-terminal region of PICH. None of these mutants eliminated BLM binding, while maintaining thread localization. Future structural studies are needed to design such key mutants.

3. The nuclesome remodeling activity of PICH is quite weak compared to CHD1 and RSC. Does PICH have nucleosome-stimulated ATPase activity? Can authors try other nucleosome remodeling assays? The bona fide SNF2 remodeling enzymes often contain other domains that interact with histones, such as CHD or bromo domains. Does PICH have any such motifs? If not, the authors may want to investigate the possibility whether PICH is a DNA helicase or translocase.

Response: There are several possible reasons for why PICH on its own is a weak nucleosome remodeling enzyme. First, PICH is a component of a multi-subunit complex, including BLM, TopoIIIalpha, RMI1, and RMI2. All these components are required to prevent micronuclei formation. Thus, only the intact complex is expected to have high nucleosome remodeling activity. Second, PICH and BLM are phosphorylated in mitosis. Phosphorylation might be required to stimulate their activity. Third, PICH might only efficiently remodel chromatin fibers that are stretched and are under tension.

PICH does not have other known chromatin binding motifs, such as chromo or bromo domains. As suggested by the reviewer, we have tested whether PICH is a bona fide DNA helicase. PICH does not catalyze the unwinding of a replication-fork-like DNA duplex. Nor does it stimulate the DNA unwinding activity of BLM. Thus, PICH is not a true DNA helicase and does not catalyze DNA strand separation. These results are included in Figure 8C.

4. The authors should investigate whether the ATPase point mutant of PICH (K128A) can rescue other phenotypes of siPICH cells, such as BLM localization to anaphase threads, and absence of histone H2B on threads, etc. This will reveal whether the remodeling activity of PICH is required for loading of BLM to the bridges.

Response: This is a great suggestion. As shown in Figure 8B, PICH K128A could not rescue the micronuclei phenotype of PICH RNAi cells. In Figure S7, we show that PICH K128A is deficient in thread localization, indicating that the ATPase activity of PICH is required for thread formation. Expectedly, PICH K128A did not restore BLM staining.

5. In Figure 1B, the Western blotting of PICH showed high background in the 6 lanes on the right. The major bands do not appear to have the same mobility as those on the left, and it is not clear to me which band is PICH.

Response: The gel did not run straight on the right side. The positions of the hyper- and hypo-phosphorylated PICH bands are now indicated.

6. Figure 3D needs mock-treated cells as controls.

Response: We thank the reviewer for pointing out this important omission. The percentage of CREST-positive micronuclei did not change between control and PICH-/BLM-RNAi cells. All these cells contained about 50% micronuclei that were CREST-positive. Only the numbers of micronuclei increased in PICH-/BLM-RNAi cells. We have simply mentioned this fact in the text, and removed the original Figure 3D, as it is not informative.

7. The authors claimed (Page 13, 2nd paragraph) that in BLM-RNAi cells, about 44% PICH-positive threads contained histones. What is the percentage in mock-treated cells? Can authors verify this result using BLM patient-derived cell line and the same line complemented by exogenous BLM?

Response: None (0%) of the PICH threads in control cells contained histones or DAPI. We have tabulated these data in Figure 6D. As suggested by the reviewer, we have verified this result using a patient-derived BLM-deficient cell line (PSNG13) and the same line complemented by ectopic BLM (PSNF5). These results are included in Figure 7. Some PICH threads in the BLM-

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complemented PSNF5 line contained DAPI. This could be due to the incomplete rescue of the BLM deficiency.

8. In Figure 7, the authors interpreted their findings that PICH and CHD1 moved nucleosomes toward the central positions on DNA. They stated that the result is consistent with the fact that the helicase domains of both PICH and Chd1 belong to the SNF2 family of helicases. This statement is somewhat midleading because RSC is also a SNF2 helicase, even though it mobilizes nucleosomes to the border positions.

Response: We thank the reviewer for pointing out this fact. The misleading statement has been removed.

Referee #2:

1. The first paragraph of the results is a repetition of recently published results (Hubner et al. Chromosoma, 2010), so that this could be removed or dramatically shortened.

Response: As suggested, the data showing that PICH is not required for the spindle checkpoint have been removed.

2. Although the authors suggest that histone remains at the DNA threads in PICH RNAi cells, a control is lacking in this experiment. Do the authors mean that the ratio of the amount of histone per DNA thread is increased by PICH RNAi? If so, quantitative data with full controls should be presented. The presented data may represent simply that the number of threads is increased in PICH or BLM RNAi cells.

Response: We show that the number of PICH threads that are positive for histones and DAPI increases in BLM-RNAi cells. These PICH-/histone-positive threads are not observed in control cells. We are making a binary statement here. The thread is either histone-positive or histone-negative. We are not making statements about the ratio of the amount of histone per DNA thread.

3. It is interesting that the PICH K128A mutant loses the nucleosome sliding ability in vitro as well as the ability to resolve DNA threads in vivo. However, since this in vitro activity of PICH is marginal and the in vivo activity is not supported (see comment 2), it is premature to conclude that PICH acts as a chromatin remodeling enzyme to resolve DNA threads. I would like to see the canonical helicase assay rather than the nucleosome sliding assay.

Response: As suggested by the reviewer, we have tested whether PICH is a bona fide DNA helicase. PICH does not catalyze the unwinding of a replication-fork-like DNA duplex. Nor does it stimulate the DNA-unwinding activity of BLM. Thus, PICH is not a true DNA helicase and does not catalyze DNA strand separation. These results are included in Figure 8C.

There are several possible reasons for why PICH is a weak nucleosome remodeling enzyme. First, PICH is a component of a multi-subunit complex, including BLM, TopoIIIalpha, RMI1, and RMI2. All these components are required to prevent micronuclei formation. Thus, only the intact complex is expected to have high nucleosome remodeling activity. Second, PICH and BLM are phosphorylated in mitosis. Phosphorylation might be required to stimulate their activity. Third, PICH might only efficiently remodel chromatin fibers that are stretched and are under tension.

4. Considering that both PICH and BLM helicases are required to resolve DNA threads at centromeres, it would be nice to analyze these proteins in parallel in Fig. 6B and 6D.

Response: We could not analyze whether PICH was required to keep histones from associating with these threads, as there were no other markers that could mark these threads in the absence of PICH. We could do so for BLM RNAi cells, as these cells still contained PICH threads. We have shown in Figure 4C and 4D that PICH is indeed required for resolving anaphase threads.

5. IP products of PICH should be tested in vitro by mixing with BLM (Fig. 6D). Similarly, BLM IP could be analyzed by mixing with PICH. Usage of mutant proteins will further strengthen the

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conclusion.

Response: This is a great suggestion. We have indeed attempted to do this. Unfortunately, the nucleosome remodeling assay does not work well with enzymes tethered to antibody beads. We have so far failed to develop efficient ways to elute the native complex off affinity beads.

6. In Fig 6B, I wonder whether BLM localizes at the threads in siPICH+GFP-PICH-K128A cells.

Response: This is a great suggestion. We have shown that PICH-K128A does not efficiently form threads. These results are now included in Figure S7. As expected, BLM does not localize to threads in these cells.

Referee #3

1. p4 Introduction "Because RNAi-depletion or chemical inhibition of topoisomerase..." should be amended to "Because depletion or chemical inhibition of topoisomerase..." since in the papers cited various methods (RNAi, but also genetic modification through gene targeting) are used to deplete the protein.

Response: As suggested, we have revised this statement to "Because inactivation of topoisomerase…".

2. This manuscript reports that PICH is not required for the spindle checkpoint. This finding is consistent with the report by Hubner et al 2010 and attention should be drawn to this earlier publication not only in the introduction, but also in the relevant results section (p. 6).

Response: As suggested by the other reviewers and by the editor, we have removed the data showing PICH is not required for the spindle checkpoint.

3. The manuscript goes onto to report the binding of PICH and BLM, including expression of the recombinant proteins in insect cells. This association appears to involve hyperphosphorylated PICH and while detectable in G1/S, is stronger in M phase. Truncated forms of PICH suggest that the BLM-interacting domain resides in the C-terminal region, although contribution of the N-terminal region to this interaction could not be fully investigated due to failure of this domain to express well in HeLa cells. While PICH can be localised both in the cytoplasm and nucleus, BLM is largely restricted to the nucleus and nucleolus and shows poor colocalisation with PICH in interphase cells. They go onto to show that, unlike BLM deficiency, depletion of PICH has no effect on SCE levels. They conclude that PICH is not required for BLM function in interphase. This aspect of the study is interesting and novel.

Response: We thank the reviewer for the positive comment.

4. Consistent with earlier reports (Baumann, 2007; Chan, 2007) they show that in anaphase cells PICH localises to DNA threads that are also coated with BLM. Some of these threads are ultrafine, being undetectable by DAPI staining and lacking histones. They confirm that the localisation of BLM to these threads is PICH-dependent, which is consistent with Chan et al 2007, who reported that BLM associates with PICH-coated anaphase threads at a later stage. They also confirm that many (although not all) of the ultrafine threads connect centromeres. In this m/s they go on to show that depletion of PICH, like depletion of BLM, increases the incidence of micronuclei, many of which are CREST-positive. Moreover, live imaging of cells expressing H2B:mCherry has also revealed that depletion of either PICH or BLM produces chromatin anaphase bridges.

Response: This is a very cogent summary of what we have shown.

5. Using HeLa cells expressing H2B-GFP the authors report that they see an increase in the number of anaphase cells that have PICH-associated chromatin bridges in BLM-deficient cells compared with BLM normal HeLa cells. The implication, from the data in Figures 4 and 5, is that in HeLa ~50% of anaphases have PICH threads, and only a small proportion (~1/6th) of these anaphase cells have chromatin-associated PICH fibres. In BLM-depleted HeLa ~48% of anaphases have

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PICH-coated connections (similar to BLM-normal HeLa cells), but of these about half have chromatin-associated fibres. The authors state that there is no increase in the number of PICH threads per anaphase cell, which implies that the numbers of PICH-associated anaphase connections that lack detectable H2B decrease in BLM-depleted cells compared to BLM-normal HeLa anaphases (the difference being replaced by histone-associated fibres). They state that in BLM-depleted cells about half of PICH fibres have H2B.

I would like to see this data presented more fully and with greater clarity (with inclusion of data on numbers of PICH-positive threads containing histones for the "normal" HeLa cells, and data on the actual numbers of connections per anaphase cell under the various conditions, and the numbers in early compared with late anaphases).

Response: As suggested, we have tabulated our results more fully in Figure 6D. Because the two major segregating masses of chromosomes overlapped during early anaphase, it was difficult to ascertain whether PICH threads contained histones at this stage or they simply overlapped with the bulk of histone signals by chance. For this reason, we only analyzed PICH threads in late anaphase to determine if they contained histones.

6. The authors state depletion of BLM from HeLa cells does not have any effect on the either the percentage of anaphase cells that contain PICH threads, or the average number of PICH threads per cells. This contrasts sharply with the findings of Chan et al. 2007 who reported, in BLM-negative cells, a significantly increased frequency of DAPI-negative PICH DNA threads (both the average number per anaphase cell, and the total number of PICH thread-positive anaphase cells). Chan et al. argued that this suggested that BLM may play a role in suppressing the accumulation of DAPI-negative PICH DNA threads in anaphase.

No reference to this difference in findings is made in the current manuscript. How do the authors account for this crucial difference? According to this earlier study, in the absence of BLM, the numbers of ultrafine, DAPI-negative PICH-associated DNA threads actually increased. This would be counter to the model proposed in this manuscript, which has the PICH-BLM complex involved in removing/preventing histone incorporation along these connecting threads.

Response: To directly address the reviewer's concern, we examined the PICH threads in BLM-deficient cells (PSNG13) and in stable transfectants of BLM-deficient cells that expressed a functional BLM transgene (PSNF5), the same cell lines as used by Chan et al. Consistent with Chan et al., PSNF5 cells contained fewer PICH threads per anaphase cells (Figure 7B). By contrast, there was no appreciable difference in the number of PICH threads between control and BLM-RNAi HeLa cells (Figure 6D). The underlying reason for this discrepancy was not clear. One possibility was that BLM depletion in HeLa cells was incomplete. The residual BLM was sufficient to promote the resolution of some PICH-positive threads, which failed to accumulate in BLM-RNAi cells.

Importantly, the BLM-deficient cells (PSNG13) contained more and higher percentage of PICH threads that were positive for DAPI (Figure 7B,C), as compared to PSNF5 cells, confirming the results in BLM-RNAi HeLa cells. We could not co-stain these cells with both antibodies against PICH and histones, as the commercially available monoclonal antibodies against bulk histones did not work in IF. On the other hand, all PICH-/DAPI-positive threads in BLM-RNAi cells contained H2B-GFP, and vice versa. Thus, the PICH-/DAPI-positive threads in BLM-deficient PSNG13 cells likely contained histones.

7. Finally the manuscript investigates the nucleosome remodeling activity of PICH. The K125A mutant disrupts its activity, consistent with the earlier work of Baumann et al. 2007. BLM was found not to have any effect on this in vitro assay. These findings, on PICH's influence on nucleosome positioning, are interesting and novel.

Response: We thank the reviewer for the positive comment.

Minor points:

8. Figure S2 - labelling error in (B).

Response: The labeling error has been corrected.

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9. Lack of scale bars for most microscopy images.

Response: Scale bars have been added.

10. The title over-interprets the data presented. Evidence for PICH limiting histone incorporation is preliminary. PICH and BLM may promote resolution of anaphase centromeric DNA threads by limiting their association with histones.

Response: As suggested by the reviewer, we have changed “histone incorporation” to “histone association” in the title and throughout the text.

11. In the Discussion (p.19) the authors state that "Unexpectedly, PICH/BLM-RNAi cells contain thin histone/CENP-A-positive chromatin threads ..." . In the data presented no CENP-A-specific antibodies have been used, only CREST antisera. Therefore the authors cannot assume that these fibres contain CENP-A.

Response: We have changed “CENP-A” to “CREST”.

2nd Editorial Decision 15 June 2011

Thank you for submitting your revised manuscript on PICH/BLM cooperation for our consideration. It has now been assessed once more by the original referees 1 and 2. While referee 1 has no more objections agains publication in The EMBO Journal, referee 2 still retains some reservations, concerning your response to one of the most significant original concerns about PICH threads with or without histones. In this respect, the referee requests the inclusions of examples from the raw image data in addition to the currently presented tabulated results (please see below for detailed explanation in his/her comments).

I would therefore like to return the manuscript to you once more for a final round of modification, in which I hope you will be able to clarify this remaining issue (please also try to take the referees cautionary notes on qualifying the conclusions from the results into account).

In addition, before eventual acceptance there are also a few editorial concerns that need to be taken care of:

- for the supplementary information, please upload all text and figure (i.e. non-movie, non-data set) material as a single PDF document (for our readers' convenience). Make sure to remove all supplementary figure legends etc from the main manuscript.

- please amend the main manuscript with a brief statement on the individual contributions of each author on the paper.

Should you have any questions or need for discussion in relation to this additional round of revision - in particular with respect to the remaining points raised by referee 2 - please don't hesitate to contact me directly.

Best regards,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

The authors have done an excellent job of performing recommended experiments and addressing my comments. The new data make the manuscript much more convincing than the previous version. I

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recommend it for publication in EMBO.

Referee #2 (Remarks to the Author):

The authors have addressed some of my concerns. However, I still have one strong reservation about their conclusion or proposal. In response to my comment 2 (and referee 3's comment 5), they state that 'about 44% PICH-positive threads contained histones whereas none of the PICH threads in control cells contained histones' without showing the raw data. It is absolutely necessary to show examples of PICH threads with DNA but not H2B (control cells) in comparison with BLM-RNAi cells. Currently, they only show examples of siBLM cells, which show PICH threads with DNA and H2B in Fig 6C. Without clarifying this point, I cannot accept the author's hypothesis that PICH or BLM acts by limiting histone association (title and abstract). At least, the word 'limiting histone association' should be removed from the title. Based on the data presented in the revised manuscript (especially and figure 6A and new figure 8C), it is more likely that PICH acts in thread resolution largely by recruiting an authentic effector, BLM helicase. The weak nucleosome remodeling activity of PICH might help this recruitment, which is supported by new figure S7 and figure 8E.

2nd Revision - authors' response 16 June 2011

Referee #2:
The authors have addressed some of my concerns. However, I still have one strong reservation about their conclusion or proposal. In response to my comment 2 (and referee 3's comment 5), they state that 'about 44% PICH-positive threads contained histones whereas none of the PICH threads in control cells contained histones' without showing the raw data. It is absolutely necessary to show examples of PICH threads with DNA but not H2B (control cells) in comparison with BLM-RNAi cells. Currently, they only show examples of siBLM cells, which show PICH threads with DNA and H2B in Fig 6C. Without clarifying this point, I cannot accept the author's hypothesis that PICH or BLM acts by limiting histone association (title and abstract). At least, the word 'limiting histone association' should be removed from the title. Based on the data presented in the revised manuscript (especially and figure 6A and new figure 8C), it is more likely that PICH acts in thread resolution largely by recruiting an authentic effector, BLM helicase. The weak nucleosome remodeling activity of PICH might help this recruitment, which is supported by new figure S7 and figure 8E.

Response: As suggested by the reviewer, we have included images of control cells to show that PICH threads in these cells do not contain histones or DAPI in Figure S6A. We note that even though these threads contain DNA, they cannot be stained with DAPI. This result is entirely consistent with earlier studies from the Hickson and Nigg labs. Our results clearly demonstrate that more PICH threads contain histones when BLM is compromised, suggesting that BLM has a function in regulating PICH. We stress that our results and our model do not exclude a role of BLM as an effector of PICH. Although we have shown that PICH recruits BLM to threads, we do not have evidence to indicate that BLM directly resolves these threads (especially those with catenated DNA) through its helicase activity. We have changed the title to more readily accommodate such a possibility. We have added a sentence in the Discussion to further clarify this point: “Our model is not mutually exclusive with the possibility that BLM as a part of this complex directly resolves certain aberrant DNA structures in anaphase threads, such as incompletely replicated DNA or recombination intermediates.”.

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