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Rif1 provides a new DNA-binding interface for the Bloom syndrome complex to maintain normal replication

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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 29 April 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three expert reviewers, whose comments are copied below. As you will see, these referees all consider your discovery of physical and functional interactions between vertebrate Rif1 and BLM helicase potentially interesting and thus in principle suited for publication in The EMBO Journal. Nevertheless, there are also still a number of important concerns that would need to be adequately addressed before publication may be warranted. Many of the main concerns in this respect pertain to the conclusiveness of the experimental evidence and the decisiveness of the support for the derived interpretations. As these issues are clearly delineated in the enclosed reports, I am not going to repeat them in detail here; however, I should stress that while for a number of these points additional discussion or toning down of conclusions may be sufficient, other conclusions would likely have to be supported by further and more decisive experimental data so as to avoid undermining the main message of the paper (this holds especially for some of the criticisms of referee 2). In addition, there are also various issues with the presentation, e.g. the inclusion or explanation of data/experiments currently 'not shown', the moving of the somewhat peripheral yeast data into the supplementary section, and an expansion of the current minimalist 'Material & Method' section to a more standard format (we can be flexible should length restrictions be a concern).

In summary, should you be able to satisfactorily address the main issues and improve the manuscript along the lines suggested by the reviewers, then we should be able to consider a revised manuscript

further for publication. Please be however reminded 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 experimental and editorial points raised at this stage. Finally, when preparing your letter of response, please also bear in mind that this 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). In any case, please do not hesitate to get back to us should you need feedback on any issue regarding your revision.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

Review of 'Rif1 provides an essential DNA-binding interface for the Bloom syndrome complex to maintain normal replication' by Xu et al.

This study identifies Rif1 as a novel component of the BLM-Topo 3 -RMI1-RMI2 complex. In yeast, Rif1 functions to regulate telomere length but such a function has not been observed in mammalian cells. Instead, mammalian Rif1 is known to have roles in repairing stalled DNA replication forks. The authors propose that Rif1 and the BLM complex act in concert to repair stalled forks. They show that Rif1 interacts with the BLM complex via its C-terminal domain. This C-terminal domain also interacts with DNA, preferring Holliday junctions and replication fork structures over double- or single-stranded DNA. Interestingly, this C-terminal domain is not present in yeast Rif1, which likely explains the differences seen in the yeast and mammalian studies. There is a lot of data, much of which is of high quality. The data generally support the authors' conclusions.

Comments:

On page 7, the acronym ICL was used before it was defined.

Also on page 7, the word "time" is missing from the sentence: "We next examined whether recruitment of BLM and Rif1 occurs at the same [time] and depends on each other."

The title of Figure 3, "Rif1 and BLM work in the same pathway in chicken DT40 cells to resist replication stress induced by several replication inhibitors", is inaccurate considering that it is only true for FUdR.

In Figurd 7C, no Rif1 foci are seen even though the authors say that they form similar to WT when quantitated. Perhaps they have a better picture to represent the data?

Since sgs1 is synthetic lethal with both mus81 and slx4 , the authors use the lack of synthetic lethality between rif1 and mus81 or slx4 (data not shown) as evidence for Sgs1 and Rif1 not acting in the same pathway in yeast. However, since it is not known whether this genetic interaction exists in mammals, their observation is inconclusive. However, the evidence for the lack of functional conservation between yeast and metazoan Rif1 is otherwise strong, so my suggestion would simply be to remove that section from the Discussion.

One big question is whether or not Rif1 affects the activity of the BLM-Topo 3 complex, which is

known to resolve double Holliday junctions. The authors mention in the Discussion that they failed to detect any stimulation of BLM activities by Rif1 (data not shown). It would be helpful if the authors could elaborate on this statement. At the very least it would quite informative to know which activities were tested.

Unfortunately, the genetic interaction between Rif1 and BLM is very confusing since the authors make different conclusion based on different kinds of DNA damaging agents used. Also, since the yeast homologues are different, perhaps a table summarizing their results, and the yeast results along the epistatic relationship between them would be extremely helpful for the reader. Finally, it is not clear why there would be different epistatic relationships and the differences should be addressed in the Discussion.

Referee #2 (Remarks to the Author):

In the current manuscript Xu et al pursue two experimental lines: characterization of interactions between Rif1 protein and the BLM complex and analysis of the Rif1 functions in DNA repair and homologous recombination. While the first line largely succeeds by providing convincing evidence that Rif1 represents a novel component of the BLM complex, the second line does not advance far enough in understanding of the Rif1 specific functions. Nevertheless, this is interesting work which deserves publication, provided that the concerns indicated below are addressed.
1. The authors claim in the title and throughout the text (e.g. P. 17, 19) that Rif1 provides an essential DNA-binding interface for the BLM complex. There are no data in the mns that would support this claim. BLM alone can bind DNA and shows preference for HJs. The text and the title need to be changed.
2. The statement that BLM acts upstream of Rif1 (for instance, P. 8, P. 9 and elsewhere) is an overinterpretation. The data are insufficient for this conclusion. It also conflicts with the title statement (that is also unsustainable in its own turn). Remove.
3. There are contradictory data on the expression level of Rif1 in the absence of BLM. The authors need to solve this issue experimentally; hypothesizing is insufficient. More cell lines need to be tested. The Western blot quantification needs to be repeated multiple times. If indeed, the level of Rif1 expression is decreased in BLM- cells, the reported effect of BLM on the kinetics of Rif1 recruitment is compromised.
4. What is the evidence that the BLM complex is stable under gel-filtration conditions?
5. The data on yeast (P. 15) are not directly relevant to the main line of the paper. Move to the supplement.
6. Page 6. Show "unpublished data in the Supplement"
7. Page 6. Last sentence of second paragraph. This is an overstretch, remove this conclusion.
8. Page 7. Last sentence of second paragraph. This is conclusion contradicts to data in Figure 2E that shows Rif1 recruitment to psoralen lesions in the absence of BLM .
9. Page 7. Third paragraph - ICLs abbreviation should be provided.
10. Page 7, last sentence. Rewording is required. The biochemical data cannot show that these two proteins are recruited together.
11. Page 9, Last sentence. This conclusion is vague. Reword by specifying.
12. Page 11. Replace "prevent CldU incorporation" by "inhibit CldU incorporation"
13. Page 12. Last sentence. Vague.
14. Page 16. Last paragraph. By no mean any evidence was presented on the "main" function of Rif1.
15. Page 17. Last sentence of first paragraph. The conclusion is not justified by the experimental data.
16. P. 20. Last section of the discussion. Move third sentence to Introduction, remove the rest.
17. Add error bars to all Figures.
18. Figure 3 is unreadable because of tiny symbols. Curve colors do not match colors in the legend.
19. Figure 7. Panel A and B should show the same mutants.

Referee #3 (Remarks to the Author):

In this manuscript, Xu and colleagues identified Rif1 as a novel component of the BLM protein complex. Rif1 and BLM function in the same pathway to prevent fork stalling and to promote fork

recovery after replication stress. In addition, Rif1 and BLM are recruited to ICL sites with similar kinetics, and the recruitment of Rif1 to ICL sites depends on BLM. Rif1 interacts with the BTR complex through its conserved C-terminal domain. The C-terminal domain of Rif1 also exhibits DNA binding activity with a preference toward branched DNA, such as fork structure and HJ. A Rif1 MutAC mutant, which does not bind to DNA but is still capable of interacting with the BTR complex, cannot rescue the increased sensitivity of the rif1 cells to replication stress and shows a decrease in HU-induced focus formation.

Overall, the data presented in the manuscript are clear and well executed, though there are some issues that need to be clarified:

(1) (p.6 & Fig.1C & D) The authors showed that only a subset of Rif1 is co-purified with the BTR complex, suggesting that there are BTR-independent Rif1 complex(es). What is then the abundance of the BTR complex in Rif1 IP comparing to other non-BTR proteins that are co-IP with Rif1 (Fig.1C)?

(2) It has been shown that RecQL1 and RecQL4 are recruited to Lamin B2 Ori during normal replication initiation (Thangavel et al, 2010). Is there any other RecQ helicases being recruited to the replication origins upon HU treatment, or that this recruitment is specific to the BTR-Rif1 complex?

(3) In HeLa cells, the protein level of Rif1 is greatly reduced in the BLM knockdown cells (Fig. 1E). On the other hand, PSNG13 cells derived from a BS patient didn't show a similar Rif1 reduction (Fig. 2G). Does chicken DT40 blm-/- mutant behave similarly to PSNG13 or HeLa knockdown cells? If the latter, it would explain why the blm single mutant behave similarly to the blm/rif1 double mutant, since the blm mutant is pretty much also a rif1 mutant? Due to the fact that most of the analysis in response to replication stress were performed in DT40 cells, the issue regarding to the protein stability of Rif1 in the absence of BLM should also be addressed in DT40.

(4) The authors showed that the recruitment of Rif1 to the stalled fork and the ICL site requires BLM (Fig. 2 & Supp Fig 2). It is therefore surprising that the Rif1mutAC mutant forms a protein complex with BTR normally, yet it is not recruited to DNA damaging foci efficiently (Fig. 7). The authors concluded that the ability of Rif1 to bind to DNA directly (presumably to the fork itself) is required for the recruitment of Rif1. However, if Rif1 has high affinity to the fork DNA and this DNA binding activity allows Rif1 to be recruited to the stalled fork, it would be rather difficult to rationalize why Rif1 still needs the interaction with BTR for the recruitment to the stalled fork.

1st Revision - authors' response 22 June 2010

Response to referees' comments

Reviewer 1:
This study identifies Rif1 as a novel component of the BLM-Topo 3α-RMI1-RMI2 complex. In yeast, Rif1 functions to regulate telomere length but such a function has not been observed in mammalian cells. Instead, mammalian Rif1 is known to have roles in repairing stalled DNA replication forks. The authors propose that Rif1 and the BLM complex act in concert to repair stalled forks. They show that Rif1 interacts with the BLM complex via its C-terminal domain. This C-terminal domain also interacts with DNA, preferring Holliday junctions and replication fork structures over double- or single-stranded DNA. Interestingly, this C-terminal domain is not present in yeast Rif1, which likely explains the differences seen in the yeast and mammalian studies. There is a lot of data, much of which is of high quality. The data generally support the authors' conclusions.

Response: We thank Reviewer 1 for his positive comments.

Reviewer 1's comment 1: On page 7, the acronym ICL was used before it was defined.

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Response: This has been corrected.

Reviewer 1's comment 2: Also on page 7, the word "time" is missing from the sentence: "We next examined whether recruitment of BLM and Rif1 occurs at the same [time] and depends on each other."

Response: This has been corrected.

Reviewer 1's comment 3: The title of Figure 3, "Rif1 and BLM work in the same pathway in chicken DT40 cells to resist replication stress induced by several replication inhibitors", is inaccurate considering that it is only true for FUdR.

Response: We slightly disagree with reviewer's interpretation. Our epistasis analyses showed that Rif1 and BLM are epistatic for not only FudR, but also aphidicolin. Epistasis is defined as the phenomenon where the effects of one gene are modified by another or several other genes. In the case of aphidicolin sensitivity, the effect of Rif1 mutation is partially suppressed by BLM mutation. The fact that BLM is needed for Rif1-/- cells to exhibit sensitivity is evidence that to two genes work in this pathway. That is, BLM is (at least partially) epistatic to Rif1.

To address the reviewer's comments, we changed the title to "Rif1 and BLM function in the same pathway in chicken DT40 cells to resist replication stress induced by FUdR and aphidicolin". We think that the new title is more accurate than the old one.

Reviewer 1's comment 4: In Figure 7C, no Rif1 foci are seen even though the authors say that they form similar to WT when quantitated. Perhaps they have a better picture to represent the data?

Response: We replaced the Figure 7C with a representative figure showing reduced foci formation of Rif1-MutAC.

Reviewer 1's comment 5: Since sgs1∆ is synthetic lethal with both mus81∆ and slx4∆, the authors use the lack of synthetic lethality between rif1∆ and mus81∆ or slx4∆ (data not shown) as evidence for Sgs1 and Rif1 not acting in the same pathway in yeast. However, since it is not known whether this genetic interaction exists in mammals, their observation is inconclusive. However, the evidence for the lack of functional conservation between yeast and metazoan Rif1 is otherwise strong, so my suggestion would simply be to remove that section from the Discussion.

Response: We have deleted this section from the text.

One big question is whether or not Rif1 affects the activity of the BLM-Topo 3α complex, which is known to resolve double Holliday junctions. The authors mention in the Discussion that they failed to detect any stimulation of BLM activities by Rif1 (data not shown). It would be helpful if the authors could elaborate on this statement. At the very least it would quite informative to know which activities were tested.

Response: We have included in the Discussion that we were unable to detect significant effects of Rif1 on DNA binding, replication fork reversal, or DNA-stimulated ATPase activities of BLM. As these are negative data, we did not include them in the paper.

Unfortunately, the genetic interaction between Rif1 and BLM is very confusing since the authors make different conclusion based on different kinds of DNA damaging agents used. Also, since the yeast homologues are different, perhaps a table summarizing their results, and the yeast results along the epistatic relationship between them would be extremely helpful for the reader. Finally, it is not clear why there would be different epistatic relationships and the differences should be addressed in the Discussion.

Response: We added a new Table 1 to summarize our results of both mammalian and yeast genetic studies. We agree with the reviewer that the genetic interactions between Rif1 and BLM in mammalian cells are complicated, and we do not understand its underlying mechanism. But they

appear to depend on particular types of replication stress: FUdR inhibits incorporation of thymidine nucleotides into DNA; aphidicolin directly inhibits DNA polymerase I; and HU treatment depletes dNTP pools in cells by inhibits ribonucleotide reductase. We included such a discussion in the text.

Referee #2 (Remarks to the Author):

In the current manuscript Xu et al pursue two experimental lines: characterization of interactions between Rif1 protein and the BLM complex and analysis of the Rif1 functions in DNA repair and homologous recombination. While the first line largely succeeds by providing convincing evidence that Rif1 represents a novel component of the BLM complex, the second line does not advance far enough in understanding of the Rif1 specific functions. Nevertheless, this is interesting work which deserves publication, provided that the concerns indicated below are addressed.

Response: We thank Reviewer 2 for his encouraging comments.

Reviewer 2's comment 1. The authors claim in the title and throughout the text (e.g. P. 17, 19) that Rif1 provides an essential DNA-binding interface for the BLM complex. There are no data in the mns that would support this claim. BLM alone can bind DNA and shows preference for HJs. The text and the title need to be changed.

Response: We agree with the reviewers and replaced the word "essential" with "new" in the title and text. The new title is "Rif1 provides a new DNA binding interface for the Bloom syndrome complex to maintain normal replication". We believe that this title more accurately interprets the conclusion of our paper, as our data clearly demonstrate that Rif1 is a new component of the BLM complex (Figure 1), and has a conserved DNA binding domain with preferential binding affinity to HJ and fork DNA in vitro (Figure 6).

Reviewer 2's comment 2. The statement that BLM acts upstream of Rif1 (for instance, P. 8, P. 9 and elsewhere) is an overinterpretation. The data are insufficient for this conclusion. It also conflicts with the title statement (that is also unsustainable in its own turn). Remove.

Response: We agree with the reviewer, and have removed the term "acts upstream" throughout the text. We replaced it with a statement "Rif1 is dependent on BLM for its recruitment to stalled forks (page 8) or resistance to aphidicolin-induced replication stress (page 9)". We made similar changes elsewhere.

Reviewer 2's comment 3. There are contradictory data on the expression level of Rif1 in the absence of BLM. The authors need to solve this issue experimentally; hypothesizing is insufficient. More cell lines need to be tested. The Western blot quantification needs to be repeated multiple times. If indeed, the level of Rif1 expression is decreased in BLM- cells, the reported effect of BLM on the kinetics of Rif1 recruitment is compromised.

Response: First, the Western blot quantification described in Figure 1F was done using data from at least 4 independent siRNA experiments in HeLa cells for each protein. These data are highly reproducible as shown by the small error bars in the figure. We have now added a statement in the Figure Legend to emphasize this point.
To alleviate reviewer's concerns, we included new data showing that Rif1 level was reduced in another human cell line, HEK293, when BLM or its complex components were depleted by siRNA (Supplemental Figure 4). These new data are in complete agreement with our findings in siRNA-treated HeLa cells, and support our conclusion that the stability of Rif1 is dependent on BLM and its complex components.

Second, the data (Fig.2G) showing comparable Rif1 levels in human BLM-deficient patient cells vs. its complemented cells have also been repeated 3 times, and the results are consistent (data not shown). To further ease the concerns by reviewers, we included new data showing that Rif1 level in BLM-/- chicken DT40 cells is also comparable to that of wildtype cells (Supplemental Figure 7). The new data provide more support for the notion that Rif1 level is not decreased in BLM-/- cells, and argue that the reported effect of BLM on the kinetics of Rif1 recruitment to DNA damage sites is not due to reduced Rif1 expression.

Third, we believe that it is not too surprising that the results from siRNA knockdown are different from complete gene knockout. The former method allows observation of the immediate effect of BLM depletion---the reduced Rif1 levels. In contrast, the latter technique permanently eliminates BLM, but these BLM-knockout cells need to be cultured for many generations to generate enough quantity for Western analyses. During this long-term growth, the BLM-/- cells should have ample opportunities for compensatory mechanism to develop---those that have increased Rif1 production to compensate for its reduced stability should have strong growth advantage. This is based on findings that Rif1 inactivation in mice is embryonic lethal (Buonomo et al. 2009, JCB), and rif1-/-/blm-/- double-mutant DT40 cells have slower proliferation rate compared to blm-/- single mutant cells (this study). Therefore, the reduced Rif1 expression in BLM-depleted cells may not be observed in BLM-knockout cells that have been cultured for a long time. We hope that this explains the different results in our siRNA vs. knockout studies.

Reviewer 2's comment 4. What is the evidence that the BLM complex is stable under gel-filtration conditions?

Response: We included new data (Appendix Figure 1; Referee only) to show that Rif1 co-immunoprecipitates with comparable amounts of BLM, Topo 3 and RMI1 before or after gel-filtration chromatography, indicating that BLM complex is stable under gel-filtration conditions. We did not include this figure in the manuscript, because our paper is already data-rich with 15 Supplemental Figures.

Reviewer 2's comment 5. The data on yeast (P. 15) are not directly relevant to the main line of the paper. Move to the supplement.

Response: Perhaps the reviewer did not realize that these data on yeast were already in Supplement (new Supplementary Figure 14). We further shortened the text of these results by deleting the reference to genomics data of Collins, and its relevant discussions.

Reviewer 2's comment 6. Page 6. Show "unpublished data in the Supplement"

Response: We now included these data in the new Supplementary Figure 2.
Briefly, we transfected several Flag-tagged Rif1 deletion mutants into HEK293 cells, and found that those containing the intact C-terminal domain co-immunoprecipitated with endogenous Rif1 protein (Supplemental Figure 2A, lanes 2-5), whereas the mutant lacking this domain did not (lane 6). The data indicate that Rif1 can self-associate through its C-terminal domain. To further demonstrate that the C-terminal domain can self-associate, we expressed the CII and CIII portion of the C-terminal domain as a recombinant protein in E.coli, and purified it to homogeneity (Supplemental Figure 2B). We found that this recombinant protein fractionates as a molecule of about 260 KDa on a gel-filtration column (Supplemental Figure 2C), which is more than 10-fold larger than its calculated molecular weight (about 22 KDa). The results support the notion that the C-terminal domain of Rif1 can self-associate, most likely by oligomerization.

Reviewer 2's comment 7. Page 6. Last sentence of second paragraph. This is an overstretch, remove this conclusion.

Response: We have removed this sentence.

Reviewer 2's comment 8. Page 7. Last sentence of second paragraph. This is conclusion contradicts to data in Figure 2E that shows Rif1 recruitment to psoralen lesions in the absence of BLM .

Response: We do not feel that these results are necessarily contradicting to each other. This sentence gives the most obvious interpretation of the CHIP result: i.e., BLM, Rif1 and RMI1 are all recruited to the lamin B2 replication origin, hinting that they may be recruited there as a complex, which are in complete agreement with our biochemistry evidence in Figure 1. In Figure 2E, we found that Rif1 recruitment to laser-induced ICLs can occur in the absence of BLM, but it is delayed by 30 min. It is quite possible that Rif1 recruitment to lamin B2 replication origin may also be delayed in the absence of BLM. However, we were unable to directly test this idea due to technical difficulties.

To address the reviewer's concern, we modified this sentence to the following "These data

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are consistent with the findings that hRif1 is a component of the BLM complex (Figure 1), and imply that hRif1 may be recruited to the stalled forks at the replication origin as part of this complex."

Reviewer 2's comment 9. Page 7. Third paragraph - ICLs abbreviation should be provided.

Response: This has been added.

Reviewer 2's comment 10. Page 7, last sentence. Rewording is required. The biochemical data cannot show that these two proteins are recruited together.

Response: This has been reworded. The new statement is "These results support the biochemical data that significant fractions of BLM and hRif1 are present in the same complex (Fig. 1), and imply that they may be recruited to ICLs together as a complex."

Reviewer 2's comment 11. Page 9, Last sentence. This conclusion is vague. Reword by specifying.

Response: This has been reworded to be more specific. The new statement is "Together, these data suggest that Rif1 and BLM work in the same pathway to resist replication stress induced by FUdR and aphidicolin, but not HU. "

Reviewer 2's comment 12. Page 11. Replace "prevent CldU incorporation" by "inhibit CldU incorporation"

Response: This has been replaced.

Reviewer 2's comment 13. Page 12. Last sentence. Vague.

Response: This has been reworded. The new statement is "The results suggest that the C-terminal domain is the minimal domain required for association with BLM, whereas other regions of Rif1, such as the middle non-conserved region, can enhance this association."

Reviewer 2's comment 14. Page 16. Last paragraph. By no mean any evidence was presented on the "main" function of Rif1.

Response: We removed the word "main function". The new statement is "Here we present evidence that Rif1 is a new component of the BLM complex and it cooperates with BLM to prevent accumulation of stalled replication forks."

Reviewer 2's comment 15. Page 17. Last sentence of first paragraph. The conclusion is not justified by the experimental data.

Response: This has been reworded. The new statement is "The interactions between Rif1 and these DNA structures may augment the ability of the BLM complex to process DNA intermediates generated during recovery of stalled replication forks."

Reviewer 2's comment 16. P. 20. Last section of the discussion. Move third sentence to Introduction, remove the rest.

Response: This has been done.

Reviewer 2's comment 17. Add error bars to all Figures.

Response: We have added error bars to Figure 2D, 2F, 3A-3D, 4B, 7D, and 7F. I should mention that the error bars in some graphs have similar or smaller sizes compared to the squares, triangles, or circles that illustrate the data points, so that the error bars may not be that obvious in these situations (For example, Figure 3A).

Reviewer 2's comment 18. Figure 3 is unreadable because of tiny symbols. Curve colors do not match colors in the legend.

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Response: This Figure was modified. We made the symbols bigger and changed some curve colors.

Reviewer 2's comment 19. Figure 7. Panel A and B should show the same mutants.

Response: We have repeated these experiments for all the mutants using the same order. Because of the space limitation in Figure 7, we showed the Southwestern data in Figure 7A, and gel-shift data in 7B (quantification) and Supplemental Figure 13 (raw image data).

Referee #3 (Remarks to the Author):

In this manuscript, Xu and colleagues identified Rif1 as a novel component of the BLM protein complex. Rif1 and BLM function in the same pathway to prevent fork stalling and to promote fork recovery after replication stress. In addition, Rif1 and BLM are recruited to ICL sites with similar kinetics, and the recruitment of Rif1 to ICL sites depends on BLM. Rif1 interacts with the BTR complex through its conserved C-terminal domain. The C-terminal domain of Rif1 also exhibits DNA binding activity with a preference toward branched DNA, such as fork structure and HJ. A Rif1 MutAC mutant, which does not bind to DNA but is still capable of interacting with the BTR complex, cannot rescue the increased sensitivity of the rif1 cells to replication stress and shows a decrease in HU-induced focus formation.

Overall, the data presented in the manuscript are clear and well executed, though there are some issues that need to be clarified:

Response: We thank this reviewer for his positive comments.

Reviewer 3's comment (1) (p.6 & Fig.1C & D) The authors showed that only a subset of Rif1 is co-purified with the BTR complex, suggesting that there are BTR-independent Rif1 complex(es). What is then the abundance of the BTR complex in Rif1 IP comparing to other non-BTR proteins that are co-IP with Rif1 (Fig.1C)?

Response: We have quantitatively depleted the BTR complex from HeLa nuclear extract with a BLM antibody, and found about 50% of Rif1 was co-depleted (New Supplemental Figure 3). This suggests that about 50% of Rif1 is associated with the BTR complex, whereas the other 50% is not. We have included these data and discussion in the manuscript.

Reviewer 3's comment (2) It has been shown that RecQL1 and RecQL4 are recruited to Lamin B2 Ori during normal replication initiation (Thangavel et al, 2010). Is there any other RecQ helicases being recruited to the replication origins upon HU treatment, or that this recruitment is specific to the BTR-Rif1 complex?

Response: These data already were shown in Thangavel et al, 2010 and all five RecQ helicases are recruited to the replication origins upon HU treatment.

Reviewer 3's comment (3) In HeLa cells, the protein level of Rif1 is greatly reduced in the BLM knockdown cells (Fig. 1E). On the other hand, PSNG13 cells derived from a BS patient didn't show a similar Rif1 reduction (Fig. 2G). Does chicken DT40 blm-/- mutant behave similarly to PSNG13 or HeLa knockdown cells? If the latter, it would explain why the blm single mutant behave similarly to the blm/rif1 double mutant, since the blm mutant is pretty much also a rif1 mutant? Due to the fact that most of the analysis in response to replication stress were performed in DT40 cells, the issue regarding to the protein stability of Rif1 in the absence of BLM should also be addressed in DT40.

Response: We analyzed the Rif1 protein level in blm-/- DT40 cells and found that it is comparable to that wildtype cells (new Supplemental Figure 7). The results are consistent with findings from human BLM patient cells and its complemented version, but are different from those of siRNA-treated HeLa cells. To verify the siRNA findings, we depleted BLM complex from another human cell line, HEK293, and observed similar reduction in Rif1 levels (new Supplemental Figure 4). Thus, reduced Rif1 expression can only be detected after acute depletion of cellular BLM by siRNA,

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but not when BLM gene is permanently inactivated. We believe that in the latter case, blm-/- cells may have used compensatory mechanisms to increase Rif1 production to overcome the growth disadvantage conferred by low expression of Rif1. The evidence for this is that Rif1 inactivation in mice is embryonic lethal (Buonomo et al. 2009, JCB), and rif1-/-/blm-/- double-mutant DT40 cells have slower proliferation rate compared to blm-/- single mutant cells (this study). Therefore, the reduced Rif1 expression in BLM-depleted cells may not be observed in BLM-knockout cells that have been cultured for a long time. We hope that this explains the different results in our siRNA vs. knockout studies.

Reviewer 3's comment (4) The authors showed that the recruitment of Rif1 to the stalled fork and the ICL site requires BLM (Fig. 2 & Supp Fig 2). It is therefore surprising that the Rif1mutAC mutant forms a protein complex with BTR normally, yet it is not recruited to DNA damaging foci efficiently (Fig. 7). The authors concluded that the ability of Rif1 to bind to DNA directly (presumably to the fork itself) is required for the recruitment of Rif1. However, if Rif1 has high affinity to the fork DNA and this DNA binding activity allows Rif1 to be recruited to the stalled fork, it would be rather difficult to rationalize why Rif1 still needs the interaction with BTR for the recruitment to the stalled fork.

Response: Our data in Figure 2 and 7 suggest that both BLM association and DNA binding activity are required for the optimal recruitment of Rif1 to stalled forks.
In Figure 2, we showed that Rif1 recruitment to the ICL site is delayed by 30 min in BLM-deficient cells compared to BLM proficient cells. This means that the Rif1 recruitment can still occur without BLM, albeit with a slower rate. In Figure 7, we showed that the Rif1-AC point mutant, which lacks DNA binding activity but has intact association with BLM complex, has reduced, albeit detectable, recruitment to forks stalled by HU treatment. This means that the DNA binding activity of Rif1 is important, but not absolutely essential, for Rif1 recruitment to stalled forks. In contrast, only the Rif1- C deletion mutant, which lacks both DNA binding activity and BLM association, is completely defective in its recruitment to stalled forks. The latter findings suggest that the residual recruitment of the AC mutant is likely due to its BLM association. Taken together, these results suggest that both DNA binding activity and BLM association are important for Rif1 recruitment. Here is one possible model that may explain our observations. Rif1 may be directed to the stalled forks by BLM. BLM then remodels the forks, generating intermediates such as "chicken-foot" or 4-way junction. Because Rif1 is part of the BLM complex, it may bind the remodeled fork through its CTD domain as an intramolecular reaction. This binding may allow Rif1 to be stably retained at the stalled fork and initiate subsequent repair reactions. If this DNA binding is inactivated as in the AC mutant, the association between Rif1 and the remodeled fork may become unstable so that Rif1 may be frequently dissociated. If BLM is inactivated, cells may utilize another fork remodeling enzyme (such as a different RecQ helicase) to remodel the stalled fork. However, because Rif1 is not associated with this enzyme, the binding of Rif1 to the remodeled fork becomes an intermolecular reaction, so that its recruitment becomes slower and inefficient. If both the CTD DNA binding activity and the BLM association are disrupted in Rif1 (C mutant), Rif1 can neither be directed to the fork by BLM, nor can it bind the remodeled fork generated by other remodeling enzymes through its CTD domain, so that its recruitment to the stalled forks is completely defective.
We added a new subtitle to the Results to emphasize that the optimal recruitment of Rif1 requires both its CTD-associated DNA binding activity and BLM association. We also added some of the above explanation to the Discussion, so that readers can understand our model better.

Finally, we would like to thank all three reviewers for their thoughtful comments and suggestions. We believe that our modifications based on their comments have significantly improved the quality and clarity of the manuscript. We hope that it can now be accepted for publication.

2nd Editorial Decision 12 July 2010

Thank you for submitting your revised manuscript for our consideration. It has now once more been

seen by the original referees 2 and 3, and I am pleased to inform you that they consider the manuscript adequately improved and have no more principle reservations against publication in The EMBO Journal.

Before we can proceed with formal acceptance of the paper, there are however some issues that need to be still clarified, concerning the assembly of certain figure panels:

- in Figure 5E, it appears as if the lanes 7/8 and 9 have been spliced together from different parts of the same or another gel. I realize that this may simply reflect removal of irrelevant in-between lanes from one and the same blot & scan - in this case, it would be sufficient to provide an uncropped image of the original blot, including explanations, as a supplementary figure, and to clearly indicate the splicing through a clear black separation line between the assembled lanes, accompanied by a brief explanation in the respective figure legend. Should these lanes however not stem from the same blot & exposure, I would need to kindly ask you to provide an alternative such figure (if necessary through repetition of this experiment), as I think it is important to be able to compare these bands directly in order to draw any conclusions from this experiment.

- also in Figure 6D, there appears to be a visible separation line of different background intensities running slightly diagonally through the upper half of at least the leftmost two panels. I am not sure what this may stem from, but again it is important that any digital assembly of panels be both clearly indicated (separation lines & explanations) and backed up by being able to look at the original data. Thus, please also clarify this point.

I am requesting these changes now in order to avoid potential problems arising after publication of your manuscript, and in accordance with the instructions for image preparation below and in our Guide for Authors. Therefore, I am returning the manuscript to you once more, hoping that you will be able to provide these clarifications and a re-revised manuscript with these figures corrected as soon as possible - pending satisfactory clarification of these issues, we should then hopefully be able to proceed swiftly with acceptance and production of the paper. In any case, please do not hesitate to contact me if you need any further clarifications.

Thank you very much for your understanding.

Yours sincerely,
Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #2 (Remarks to the Author):

I find the revised version suitable for publication in EMBO journal.

Referee #3 (Remarks to the Author):

The authors did a good job addressing the reviewers' questions. I recommend the current version of the manuscript for publication.

2nd Revision - authors' response 12 July 2010

Thank you for your email regarding our manuscript (EMBOJ-2010-74426R). My colleagues and I are delighted to hear that both reviewers 2 and 3 have recommended publication. I also want to

thank you for careful examination of our figures. Below is our clarification for the two figures mentioned in your email.

1. In Figure 5E, lanes 7-9 are spliced and assembled from the same immunoblotting images under the same exposure. Per your request, we included uncropped original images as the new Supplemental Figure 14 to illustrate where the splicing and assembly occur. We added black lines to separate lanes 8 and 9 to clearly mark the assembled lanes. We also added a statement in Figure 5E legend to indicate this.

2. For Figure 6D, I attached the original images from the phosphoimager (Appendix Figures 1 and 2). As you can see in Appendix Figure 1, which includes images for the two left panels of Figure 6D, the visible separation line mentioned in your email is present in these original images. It runs across from the left of the screen all the way to the right, even in area where there is no gel. We do not understand where it is from. One likely explanation is that the phosphoimager screen may not have been completely "bleached" before the current experiment; and this line may represent the border of an image from an earlier experiment that was not completely wiped out so that it shows up in the current exposure. The bottom line is that this line is not a result of image manipulation (we actually did not perform any image manipulation for this figure).

I hope that the above clarifications are satisfactory. If you have more questions, please feel free to contact me.

Acceptance letter 13 July 2010

Thanks very much for these prompt and absolutely satisfactory clarifications. We shall be happy to proceed with the publication of your manuscript now, and you will receive a formal letter of acceptance shortly.

Best regards,

Editor
The EMBO Journal

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