THE EMBO JOURNAL

Manuscript EMBO-2012-84004

Ubiquitin-dependent recruitment of the Bloom Syndrome helicase upon replication stress is required to suppress homologous recombination

Shweta Tikoo, Vinoth Madhavan, Mansoor Hussain, Edward S. Miller, Prateek Arora, Anastasia Zlatanou, Priyanka Modi, Kelly Townsend, Grant S. Stewart, Sagar Sengupta

Corresponding author: Sagar Sengupta, National Institute of Immunology

Review timeline:

Submission date:	22 November 2012
Editorial Decision:	16 January 2013
Revision received:	13 April 2013
Accepted:	02 May 2013

Editor: Hartmut Vodermaier

Transaction Report:

(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 16 January 2013

Thank you again for submitting your manuscript on BLM ubiquitylation for consideration by The EMBO Journal, and please excuse the unfortunate delay in its evaluation incurred during this very busy time of the year. We have now finally received the reports from three expert referees, as well as additional input from an Editorial Advisory Board member, and I am happy to inform you that in light of the principle interest expressed by all of them, we should be able to consider an adequately revised version of the manuscript further for publication. As you will see from the reports copied below, there are nevertheless a number of important concerns that will need to be satisfactorily addressed, and eventual acceptance of the study will fully depend on the completeness and diligence of your responses to both the referees' and the editors' concerns during the single round of major revision that our policies allow for. In particular, it will be essential to address the following points:

a) ruling out general inactivating effects of the BLM ubiquitylation site mutants (referee 2)

b) including clear quantification instead of only representative images for the cell biological/microscopy data (see referee 1 point 2) in all relevant figures.

c) improving the quality of several of the Western Blot data panels (see referee 1 point 3), also regarding exposure and controlled loading. Furthermore, make sure to show larger sections of the blots, as many of them (e.g. Figure 2D, Figure 4A/B) are cropped far too close to the bands/regions of interest.

d) providing unprocessed source data files for the various electrophoretic gels and blots in this case, to complement the required image improvement efforts. We would ask for a single PDF/JPG/GIF file per figure comprising the original, uncropped and unprocessed scans of all gel/blot panels used in the main and supplementary figures. These should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. A ZIP archive containing these individual files can be uploaded upon resubmission (selecting "Figure Source Data" as object type) and would be published online with the article as a supplementary "Source Data" file.

e) from the experimental descriptions, it is not clear how well the key RNAi experiments have been controlled for efficiency and off-target effects. It will be essential to amend especially the RNF8/RNF168 knock-down experiments with rescue/complementation by RNAi-insensitive constructs, or at least by confirmation with an unrelated second siRNA against the same target, according to the current standards in the field.

f) finally, all referees mention a variety of concerns with the presentation of both the text and the figures, which will need to be significantly improved. This concerns introduction and rationalization of many experiments, organization of figures, discussions in the context of the literature, as well as general writing

Should you be able to decisively address these key points, and adequately answer to the various other specific comments not reiterated here, then we could consider the a revised manuscript for eventual publication in The EMBO Journal. We generally allow three months as standard revision time, and it is our policy that competing manuscripts published during this period will have no negative impact on our final assessment of your revised study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Finally, when preparing your letter of response to the editors' and the referees' comments, please also remember that this will form part of the Review Process File, and will therefore be available online to the community.

Should you have any questions regarding this decision or the revision requirements, please do not hesitate to contact me directly. I look forward to your revision.

———

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

In this manuscript, Tikoo et al report that BLM is recruited to DNA damage sites via RNF8/RNF168-dependent protein ubiquitination pathway. The authors show that RNF8/RNF168 can ubiquitinate BLM in vitro and regulate the DNA damage-induced BLM ubiquitination in vivo. Moreover, three ubiquitination sites of BLM have been mapped, which are important for targeting BLM to DNA damage sites. Meanwhile, the ubiqutinated BLM is recognized by RAP80, an Ub receptor during DNA damage response, for protein degradation. Overall, it is an interesting research project, and the authors show the convincing results to justify the conclusions. I only have some suggestions for the authors as listed:

1. It is interesting to see that RAP80 regulates the stability of BLM. However, it is not clear the role of RAP80 in the proteasome-dependent protein degradation pathway, especially during DNA damage response. I suggest that the authors carefully discuss the function of RAP80 in the discussion section. Particularly, BRCC36, the partner of RAP80, is known to digest K63-linked poly-ub chain.

2. In Figure 1, the ub-dependent recruitment of BLM to DNA damage sites is solid and convincing. However, only representative images were shown in the manuscript. If the authors can quantitatively summarize the results, it might be easier for readers to understand the data.

3. Authors might consider improving the quality of some Western blot images. It seems that there are some unequal loading issues and overexposure of the Western blot signals in Figure 2B, 2D and 5C.

4. It is not clear why individual Lys mutation could drastically reduce the BLM ubiquitination in vitro (Fig. 4A). Usually, the ubiquitination sites are interchangeable between different Lys residues at least in vitro.

Referee #2 (Remarks to the Author):

Tikoo et al presented interesting results of their studies on the role of the ubiquitin-dependent DNA damage response (Ub-DDR) in regulation of recruitment of BLM, an important caretaker tumor suppressor, to stalled replication forks.

Here, several novel and significant observations have been reported.

1. The authors show that BLM is ubiquitylated by the E3 ligases RNF8/RNF168.

2. This polyubiquitylation triggers the recruitment of BLM to sites of stalled replication forks and regulates its nuclear localization in unstressed cells.

3. RAP80 binds to BLM in ubiquitin-dependent and ubiquitin-independent manners and inhibits its proteasomal degradation.

There is one concern that needs to be addressed before the manuscript can be published in EMBO journal.

An important argument for the role of BLM ubiquitylation in DNA damage recruitment was gained from experiments with the BLM mutants in which three lysines (K105, K225, and K259), sites of ubiquitylation, were replaced with arginines. The authors demonstrated nicely that these mutations reduce significantly ubiquitylation of the protein. However, these mutations may have additional effects on the protein function. If it is the case, one of the most important arguments for the role of ubiquitylation would be compromised. Therefore, it needs to be demonstrated that the mutations do not disturb the basic activities of the protein, such as helicase.

Specific concerns:

1. The details of some experiments are not properly explained. For instance, what was the rationale for using P4D1 vs FK1 antibodies in Fig. 2. Or what was the meaning for using HU postwash conditions as shown in Fig. 1E and 6A?

2. Figures and Figure legends are not well organized. For instance, in Fig. 2 the legend for panel E precedes those for D and C. Can the figure be re-arranged to avoid this inconvenience for readers.

3. The rationale for experiments with fusion proteins BLM-H2AX and FHA domain MDC1 are extremely poorly explained. And the results of these experiments are poorly discussed as well

4. Proofreading is required. Some awkward expressions are abundant: Page 7: .."bacterially purified RNF8..."

Referee #3 (Remarks to the Author):

The authors of this study report a strong connection between DNA damage inducible ubiquitylation and the anti-recombinogenic function of the Blms helicase. Specifically, Blm is ubiquitylated at its N-terminus by RNF8 and RNF168 in association with E2 enzymes Ubc13 and UbcH5a. K63-ubiquitylation of Blm is required for its localization to HU stalled replication forks. RAP80 binds Blm and surprisingly is involved in its stabilization. This manuscript implicates this pathway in suppressing SCEs.

This is an extensive body of work and of interest to the genome integrity field. I have several minor concerns that should be addressed prior to publication.

1) Fig 1D and others show Blm in the nucleolus. Nucleolar localization is a known artifact of GFP fusion proteins. I would like to see staining with an antibody to endogenous Blm show the same localization in nucleoli before. In the absence of confirmation with endogenous staining, one cannot be certain that it is not due to overexpression of the GFP-fusion. The conclusions of this study will not be negatively impacted if nucleolar localization is not verified. The authors will only need to remove reference to this.

2) Page 14 - The authors cite RAP80 as playing an anti-recombinogenic role. They should also cite Hu and Livingston, Genes & Dev 2011, which showed similar findings to Coleman and Greenberg JBC 2011.

3) The authors suggest that RAP80 mutation in humans would have similar phenotypes to Blms mutations. A RAP80 knockout mouse has been published, (Yin et al. Cancer Res 2012). The mouse has a much milder phenotype than do Blm KO mice. This merely suggests that while RAP80 may stabilize Blm and be responsible for some of its functions, it is not essential for all Blm function. The authors should modify their discussion accordingly.

1st Revision - authors' response 13 April 2013

We are submitting the revised version of the manuscript entitled “Ubiquitin-dependent recruitment of the Bloom Syndrome helicase in response to replication stress is required to suppress homologous recombination” (EMBOJ-2012-84004). We have highlighted the changes made in the revised version in response to the reviewer’s comments. A separate document in which we have responded to each of the reviewers’ comments is also included. Finally we have responded to all the suggestions made in your decision letter with respect to the original version of the manuscript.

We hope that you will now find the manuscript suitable for publication in The EMBO Journal.

Response to the reviewers:

Reviewer 1

Query #1:

It is interesting to see that RAP80 regulates the stability of BLM. However, it is not clear the role of RAP80 in the proteasome-dependent protein degradation pathway, especially during DNA damage response. I suggest that the authors carefully discuss the function of RAP80 in the discussion section. Particularly, BRCC36, the partner of RAP80, is known to digest K63-linked poly-ub chain.

Response

We were surprised by a requirement of RAP80 in maintaining the stability of BLM in the absence of DNA damage and are therefore unclear as to the mechanism underlying this function. Whilst we feel that further experimental examination in this lies beyond the scope of this paper, as the reviewers suggests we have provided some suggestions as to how RAP80 modulates BLM protein levels in the discussion. Incidentally we have discussed that BRCC36 alone or in combination with other DUBs

(such as USP3 and OTUB1) be involved in removing BLM from sites of DNA damage following the resolution of HR intermediates (Page 16-17).

Query #2:

In Figure 1, the ub-dependent recruitment of BLM to DNA damage sites is solid and convincing. However, only representative images were shown in the manuscript. If the authors can quantitatively summarize the results, it might be easier for readers to understand the data.

Response

We thank the reviewer for pointing out this lacuna in the original version. In the revised version the quantification of the representative immunofluorescence images has been added all throughout the manuscript (Figure 1D, 1E, 5B, 5D, S1B, S1C, S4B). The numbers in the combined panel denotes the percentage of the cells which show colocalization between the two proteins. This has also been indicated in the Materials and Methods section (page 20).

Query #3:

Authors might consider improving the quality of some Western blot images. It seems that there are some unequal loading issues and overexposure of the Western blot signals in Figure 2B, 2D and 5C.

Response

The authors thank the reviewer for pointing out these issues. We have addressed these issues in the following way:

A. In Figure 2B, we have added a loading control (a Coomassie stained gel for BLM used for ubiquitylation). Unfortunately a lesser exposure for ant-BLM western was not available.
B. In Figure 2D, we have provided lesser-cropped gels and also provided a loading control for BLM.
C. For Figure 4A and 4B, based on the editor's request, we have provided lesser-cropped gels and also an additional exposure which shows the differential ubiquitylation between BLM (WT) and BLM (3K-R).
D. For Figure 5C, we have provided autorads for PML III and PML IV ubiquitylation which are of lesser exposure. Additionally we have provided the Coomassie gels for PML III and PML IV, which serve as the loading control.

Query #3:

It is not clear why individual Lys mutation could drastically reduce the BLM ubiquitination in vitro (Fig. 4A). Usually, the ubiquitination sites are interchangeable between different Lys residues at least in vitro.

Response

Whilst we agree with the reviewer's comments that sites of ubiquitylation for many proteins are often interchangeable, at least *in vitro*, this is certainly not the case for all proteins. In this respect, the ubiquitylation of FANCD2, FANCI and PCNA only occurs on a single lysine residue *in vitro* and *in vivo* and when the individual lysine is mutated then the ubiquitylation is completely abrogated. However, it has also been documented that RNF168 and RNF8 can target multiple lysines within the same protein for ubiquitylation e.g. K13 and K15 in H2A(X) or K435 and K502 in Nbs1 respectively.

Despite this, we demonstrate that loss of anyone of the 3 lysines targeted by RNF8/RNF168 causes a reduction in BLM ubiquitylation *in vitro* but that this is not completely lost until all three N-terminal lysine residues are mutated. This could indicate that either RNF8/RNF168 targets any of these lysine residues in BLM for ubiquitylation individually or in combination or as the reviewer indicates, when the primary site(s) of ubiquitylation is lost, a nearby lysine contained within a permissive structure is targeted *in vitro* albeit with a reduced efficiency. Irrespective of this, we go on to show that loss of

these sites of BLM ubiquitylation prevents BLM being recruited to sites of replication stress and as a consequence, its ability to suppress homologous recombination repair indicating that they are functionally relevant sites of post-translational modification.

Reviewer 2

General critique

An important argument for the role of BLM ubiquitylation in DNA damage recruitment was gained from experiments with the BLM mutants in which three lysines (K105, K225, and K259), sites of ubiquitylation, were replaced with arginines. The authors demonstrated nicely that these mutations reduce significantly ubiquitylation of the protein. However, these mutations may have additional effects on the protein function. If it is the case, one of the most important arguments for the role of ubiquitylation would be compromised. Therefore, it needs to be demonstrated that the mutations do not disturb the basic activities of the protein, such as helicase.

Response

We appreciate the reviewer's concern. Hence we carried out helicase assay with GST-BLM (WT) and GFT-BLM (3K) proteins. We did not see any difference in the extent of DNA unwinding effect by the two proteins. The data along with the quantitation has been incorporated in Figure S2B. The experiment has also been incorporated in the text (page 8). Reference of the protocol used for helicase assay has been included in the supplementary materials and methods.

Query #1:

The details of some experiments are not properly explained. For instance, what was the rationale for using P4D1 vs FK1 antibodies in Fig. 2. Or what was the meaning for using HU post-wash conditions as shown in Fig. 1E and 6A?

Response:

The rationale for using the P4D1 vs FK1 antibodies is that the P4D1 antibody recognizes all forms of ubiquitin, whereas FK1 only detects poly-ubiquitin chains. These two antibodies (along with FK2 in Figure 5C) were used to distinguish whether the high molecular weight species of the tested proteins (BLM/PML) observed in our ubiquitylation reactions were due to multiple sites of mono-ubiquitylation versus poly-ubiquitylation. This rationale has been incorporated in the Materials and Methods (page 20).

We have intentionally used the +HU/PW condition to show that the BLM/RNF168 colocalization (Figure 1E) and BLM/RAP80 interactions (Figure 6A, 6B) were enhanced during the stalling of the replication forks (during HU-treatment). This has been incorporated in the text (page 6 and 9-10).

Query #2:

Figures and Figure legends are not well organized. For instance, in Fig. 2 the legend for panel E precedes those for D and C. Can the figure be re-arranged to avoid this inconvenience for readers.

Response

We thank the reviewer for pointing this out. This has been rectified. The changes in the figure legends are reflected in the text (page 31).

Query #3:

The rationale for experiments with fusion proteins BLM-H2AX and FHA domain MDC1 are extremely poorly explained. And the results of these experiments are poorly discussed as well.

Response:

Again we thank the reviewer for pointing this out. We have now expanded the rationale of generating the two fusion proteins, H2AX-BLM and MDC1-FHA-BLM in page 11 of the revised manuscript.

Query #4:

Proofreading is required. Some awkward expressions are abundant: Page 7: .."bacterially purified RNF8..."

Response

The manuscript has again been proofread. In the specific instance, the expression has been changed in page 7.

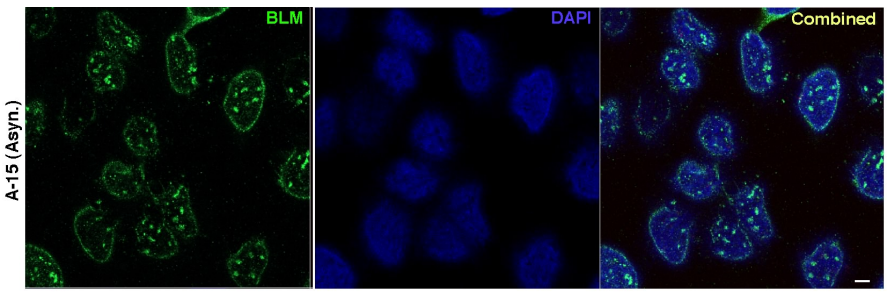
Reviewer 3:

Query #1:

Fig 1D and others show Blm in the nucleolus. Nucleolar localization is a known artifact of GFP fusion proteins. I would like to see staining with an antibody to endogenous Blm show the same localization in nucleoli before. In the absence of confirmation with endogenous staining, one cannot be certain that it is not due to overexpression of the GFP-fusion. The conclusions of this study will not be negatively impacted if nucleolar localization is not verified. The authors will only need to remove reference to this.

Response:

We agree with the reviewer's comment regarding nucleolar targeting often being an artifact of fusion with a GFP tag. However, it has been previously demonstrated that endogenous BLM also resides in the nucleoli (Sanz et al. (2000) Cytogenet Cell Genet 91:217-223; Yankiwski et al. (2000) PNAS 97:5214-9). Furthermore, BLM has also identified by mass spectrometry as a component of the nucleolar proteome (Leung et al. (2006) Nucleic Acids Res 34:D218-20). However to satisfy the reviewer's query we are including below the nucleolar staining of endogenous BLM in A-15 cells.



BLM is present in nucleolus in absence of DNA damage. A-15 cells were grown under asynchronous conditions and stained with anti-BLM antibody (A300-120A). Nuclei is stained with DAPI. Nucleolar accumulation of BLM is indicated by DAPI exclusion. Scale 5μM.

Query #2:

Page 14 - The authors cite RAP80 as playing an anti-recombinogenic role. They should also cite Hu and Livingston, Genes & Dev 2011, which showed similar findings to Coleman and Greenberg JBC 2011.

Response:

This reference has been added to the revised manuscript (page 16).

Query #3:

The authors suggest that RAP80 mutation in humans would have similar phenotypes to Blms mutations. A RAP80 knockout mouse has been published, (Yin et al. Cancer Res 2012). The mouse has a much milder phenotype than do Blm KO mice. This merely suggests that while RAP80 may stabilize Blm and be responsible for some of its functions, it is not essential for all Blm function. The authors should modify their discussion accordingly.

Response:

We appreciate the reviewer's viewpoint. In the discussion regarding the possible similarity between the clinical symptoms of Bloom Syndrome patients and those that could be hypothetically caused by a RAP80 deficiency has been suitably adjusted to include the reviewer's observation (page 15).

Acceptance letter 02 May 2013

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

Referee #1

(Remarks to the Author)
The authors have addressed my questions. I recommend this manuscript for publication.

Referee #2

(Remarks to the Author)
The authors addressed my previous concerns, I don't have any additional ones.

