Bat3 Facilitates H3 K79 Dimethylation by DOT1L and Promotes DNA Damage-induced 53BP1 Foci at G1/ G2 Cell Cycle Phases

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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 07 July 2011

Thank you for submitting your manuscript on Bat3 and DOT1L interaction to The EMBO Journal. Three referees have now assessed it, and while they find the study in principle of interest to a wide readership, they at the same time raise a considerable number of concerns which we feel would need to be satisfactorily addressed before eventual publication. On one hand, there are numerous experimental and technical concerns raised by all three referees, including requests for stronger experimental data as well as better description/explanation and stronger proof of reproducibility and significance of key results. On the other hand, especially referees 2 and 3 also raise important conceptual issues - including concerns about citation and presentation/interpretation of previous works, and the key criticism that causal links between Bat3, H3K79 methylation, and 53BP1 foci formation and DNA damage responses have yet to be decisively established (see referee 2's general comments).

I realize that the referees' serious concerns amount to a substantial list, but given that the points on the whole nevertheless appear potentially addressable, I am inclined to give you the opportunity to do so through a revised version of the manuscript. Thus, should you be able to decisively clarify the current conceptual and technical issues, and to improve also the presentation and discussion of the findings, then we should be able to consider a revised manuscript further for publication. In this respect, given the long list of request I would in fact not insist on one of the further-reaching requests of referee 2 to add more mechanistic insight into how Bat3 enhances DOT1L activity - although I realize that any data you might have to shed light on this aspects would certainly increase the impact of the study. When revising the paper, please also pay attention to editorial aspects, such as the current lack of page and figure numbers or the incompleteness of citations in the reference.
list; please also explain and clarify the statistical basis for all diagrams displayed, keeping in mind that averaging and statistical analysis should only be carried out when at least 3 biological replicates have been analyzed.

I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version. 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.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
http://www.nature.com/emboj/about/process.html

Should you have any additional question regarding this decision or your revision, please do not hesitate to contact me. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

In this manuscript the authors propose that Bat3 facilitates H3K79 dimethylation by DOT1L and recruitment of 53BP1 to DNA damage foci in G1/G2 cells. Overall, this is an interesting manuscript that could become acceptable for publication, if the comments mentioned below can be addressed.

1. Fig. 1 shows that depletion of Bat3 reduces the average number of 53BP1 foci per cell (Fig. 1A) and the degree of H3K79-dimethylation (Fig. 1B). Fig. 1A is very difficult to visualize; the authors should show the outline of the nuclei and not merge the DAPI staining with the 53BP1 foci. They also should show a bar graph of the percent of cells with more than 5 foci per cell after IR treatment. Their premise is that the average number of foci per cell is decreased, because in some cells (those in G1/G2) 53BP1 does not localize to foci. This would be evident in a bar graph showing the fraction of cells with more than 5 foci per cell after IR treatment.

2. Fig. 2A: which are the scrambled siRNA treated cells and which the Bat3-shRNA treated cells? If the cells express stable-inducible Bat3 shRNA, the authors should show the stably-transfected cells before and after induction of the Bat3 shRNA. Also as in Fig. 2A, a bar graph of the percent of cells with more than 5 foci per cell after IR treatment should be shown.

3. Fig. 2B: the synchronization of the cells needs improvement. Are the cells grown in the presence of nocodazole? Why is H3pS10 maximum at 20 hours in the shBat3 cells? At that time, synchronized U2OS cells should be in G1. Also why is there H3pS10 staining at all timepoints in the scrambled shRNA cells? I would like to see this experiment repeated in its entirety. This experiment should also be performed to support the major points of the authors (for example, if WT Bat3, but not Bat3delUBL, rescues 53BP1 focus formation; same for DOT1L WT and delUIM; see below).

4. In addition, to support the arguments of the authors, the authors should study unsynchronized cells treated with BrdU or EdU 30 minutes before fixing. This staining should allow the authors to
distinguish S phase from G1/G2 cells.

5. Fig. 2D: the images should be shown as described above for Fig. 1A.

6. Fig. 3B: the images should be shown as described above for Fig. 1A.

7. Fig. 4. Coimmunoprecipitation is not the same thing as colocalization. For colocalization, the authors need to show microscopy images. Figs 4A and 4B are not convincing. Thus, this figure should and the conclusions derived from it should be deleted in its entirety.

8. Fig. 5B: the authors should show the level of expression of the wt Bat3 and delUBL Bat3 proteins.

9. Fig. 5C: the images should be shown as described above for Fig. 1A.

10. Fig. 6A: what is NAAIRS, and NAAIRS x 3? The consensus sequence of the UIM motif should be shown, as well as the UIM sequences of a couple of crystallized motifs showing also which residues interact with ubiquitin.

11. Does addback of DOT1LdelUIM rescue 53BP1 foci after IR treatment? (Fig. 6B).

Referee #2 (Remarks to the Author):

DOT1L methylates H3K79. While one initial study suggested a role for DOT1L in formation of DNA damage induced 53BP1 foci, several more recent studies have not been able to confirm this result. In fact, most studies suggest that 53BP1 is recruited by H4K20me2. Bat3 has been linked to DNA damage checkpoints and has been shown to affect H3K4 methylation. Here the authors investigate the possible links between DOT1L and Bat3 and their role in formation of DNA damage induced 53BP1 foci.

The authors first confirm that Bat3 is required for DNA damage induced 53BP1 foci formation. The effect of Bat3 knock-down is low in G1/S arrested cells but greater after release of the cells into the cell cycle, when the levels of H4K20me2 start to drop. This leads the authors to suggest that not H4K20me2 but some other mark may be involved in 53BP1 recruitment. The authors suggest that this could be H3K79me2 (e.g. see abstract). However, no direct evidence is provided.

Data presented in the manuscript suggest that Bat3 and DOT1L bind to each other via a ubiquitin-like interaction. The binding of Bat3 to DOT1L by co-immunoprecipitation is lost upon deletion of the newly identified Ub-interaction motif of DOT1L or upon deletion of the Ub-like motif of Bat3. Reduced levels of Bat3 or mutations in the interaction motifs lead to lower levels of H3K79me2, indicating that the Bat3-DOT1L interaction is required for the methyltransferase function of DOT1L.

Although the interaction between Bat3 and DOT1L, the effect of Bat3 on H3K79me2, and the role of Bat3 in DNA damage response (DDR) is interesting, it is still unclear how Bat3 affects DOT1L function and whether Bat3 affects the DNA damage response via its interaction with DOT1L. In contrast to several statements in the paper that 'H3K79-2Me is required for IR-induced 53BP1 foci', no direct evidence is provided for a role of DOT1L or H3K79me2 in this process. Bat3 affects the DNA damage response and H3K79me2, but that does not prove that Bat3 affects the DNA damage response via H3K79me. This, together with inconsistencies between figures, inaccuracies in the text, and incorrect citation of published literature dampens my enthusiasm for this paper.

Major comments

1. The claim made in the paper and in the abstract that 'H3K79-2Me is required for IR-induced 53BP1 foci' is not supported by the data. It is inferred from phenotypes of Bat3 knock-downs or Bat3 Ubl-domain mutants. However, Bat3 has several known functions unrelated to DOT1L (such as regulation of H3K4 methylation). Therefore, the Bat3 DDR phenotypes may be unrelated to DOT1L. To investigate the role of DOT1L directly the authors should examine the phenotypes of DOT1L knock-downs or of DOT1L-UIM mutants.
2. Throughout the paper many of the statements are inaccurate. I give a few examples:
- Abstract and text: 'K79 dimethylation (H3 K79-2Me) by DOT1L influences the DNA damage response by promoting 53BP1 recruitment to DNA damage sites'. As the authors explain elsewhere, most of the studies on this topic suggest that DOT1L does not play a role in the DDR. I would say that at best the role of DOT1 is still under debate. The authors suggest that the U2OS cells investigated here may be different from HeLa cells studied previously. It would be appropriate to note that the study of Botuyan et al found no effect of DOT1L in several cell lines (DOT1L knock-down in HeLa and A549 adenocarcinoma cells, and primary DOT1L +/- MEFs).
- Discussion 'It is important to note that since over 90% of all H3 is methylated, the observed ~50% reduction in H3K79-2Me suggests that Bat3 regulates histone H3 methylation across a broad spectrum of the genome'. This makes no sense. In yeast ~90% of H3K79 is methylated. In mammals, this is much less, as can be found in several high quality mass spectrometry studies.

3. The mechanism by which Bat3 enhances DOT1L activity remains unknown. The authors could test whether the UBL domain is sufficient for Dot1 activation or whether the large C-terminal part of Bat3 is also involved. This may help to generate separation-of-function mutants. Another experiment that would be very informative is to check whether deletion of the UIM of DOT1L affects the binding of DOT1L to H3 or chromatin.

4. Abstract and text and Fig. 4B: 'Bat3 knockdown results in decreased DOT1L-H3 interaction'. The evidence to support this important claim is not very strong (Fig 4B). The bands are not very strong/straight and the overall IP (H3 signal) seems lower in the siBat3 sample. To substantiate this point, which is very important for the interpretation of the further studies, the authors should show biological replicates. The authors should also consider alternative assays to determine the effect of Bat3 on DOT1L. For example, they could do ChIP to determine DOT1L binding to target loci in a quantitative manner, and they could do immunostaining to determine whether the nuclear localization is affected (as in the CALM-AF10 expressing cells that are mentioned in the paper) in Bat3 mutants or DOT1L-UIM mutants.

5. I am concerned about the variation between experiments. For example, the Bat3 IP gives a weak DOT1L co-IP in Fig 4A and a very strong co-IP in Fig. 5.

6. Figure 2 raises many questions. Is H4K20me2 higher in shBat3? Do the shBat3 cells not progress to G1 or do they progress slower? The label suggests they stay in G2/M. This can affect several of the phenotypes studied. The plot of Fig. 2D does not seem to match with the images shown on the left. For example, at 5 h the number of foci seems much lower in the siBat3 sample, but this is not the case in the graph.

7. Figure 3. Also here in panel B the images do not seem to match the graphs. The authors should explain this discrepancy. Why is the exposure of the images different?

Other Comments:

8. Most of the figure legends do not contain enough information to understand the figures. For example, the authors need to provide more information on the number of cells/biological replicates used for the plots and they need to explain the error bars. It is not clear whether the plots 2D and 3AB contain error bars? If they do they seem very small. I guess they refer to technical replicates. Also, it is not clear how the intensities were normalized to generate the bar diagrams.

9. Abstract: 'The methyltransferase DOT1L methylates histone H3 at K79, facilitating H3 interaction with various binding partners'. Binding partners of H3K79me have not been identified yet.

10. Throughout the paper the authors focus on dimethylated H3K79. From what I understand, it has not been established which of the methylation states of H3K79 are relevant for DOT1L's functions.

11. Discussion: 'it has been clearly demonstrated that in budding yeast the 53BP1 homolog Rad9 forms foci by binding to histone H3K79 residues that are dimethylated exclusively by Dot1L'. In my opinion, there is no direct evidence for the interaction between Rad9 and methylated H3K79. Also,
which of the methylation states is involved is still unknown.

12. The binding of Dot1 to H3 is referred to as co-localization. This can generate confusion because co-localization of chromatin proteins usually refers to co-localization in the same compartment or genomic regions. Perhaps the word binding would be more appropriate.

13. Very frequently the authors do not correctly cite the published literature. In addition, published data is sometimes discussed without providing a proper reference. The authors should more accurately and more extensively refer to published work. I give a few examples:
- No citation is given for the repeated statement that ‘H3 K79-2Me levels in humans remain consistently high and do not appear to fluctuate throughout the cell cycle’.
- ‘In heterochromatin, these dimethylated residues are thought to play a role in higher-order chromatin structure where the residues may be sequestered within the nucleosomes.’ No references are given. I am not aware of studies showing that the residues may be sequestered.
- ‘correlation between ubiquitination of histone H2B at K120 in humans and K123 in yeast with enhanced H3K79 methylation (Jeltsch and Rathert, 2008; Martini et al., 2002; McGinty et al., 2008)’ Is Martini the correct citation here?
- Results: ‘Interestingly, HeLa cells display high levels of H4K20-2Me’. Please provide a reference.

Referee #3 (Remarks to the Author):

The manuscript by Wakeman et al. analyzes the mechanisms underlying the recruitment of 53BP1 at DNA damage sites. Ample evidence indicates that histone modifications play a critical role in this process and the basics of the mechanisms appear to be evolutionarily conserved. What is not clear in human cell is whether 53BP1 is recruited by H3K79 dimethylation, by H4K20 dimethylation, or both. In this manuscript the authors address this question and propose that the both modifications contribute but their individual involvement is cell cycle specific. Furthermore they propose that Bat3, a protein previously known to regulate histone H4 modification on K4, by interacting with histone H3 and the histone methyltransferase DOT1L, modulates H3K79 dimethylation. The subject of the investigation is certainly interesting and in general the manuscript is very well written, the logic of the experiments is very clear. The quality of the exhibits is somewhat variable, being better in the first half of the paper. On the other hand there are several controls that are missing and that should be included to make the paper complete and conclusive.

Major comments

1- In Figure 1 the authors show that siBat3 leads to a 50% reduction in H3K79-2me. This is the fundament for the rest of the paper. In the same experiment they should also quantitate the effect of Bat3 downregulation on H3K79 mono- and tri-methylation.
2- One of the major conclusions of this work is that H4K20-2me is important for 53BP1 foci formation during S phase, while H3K79-2me is relevant in G1 and G2, when H4K20-2me is very low. This is an interesting interpretation but needs to be supported by more direct evidence. The experiment shown in Figure 2 is based on the fact that downregulation of Bat3 causes a 50% decrease in H3K79-2me. Since Bat3 is also involved in other processes, the cell cycle dependent contribution of H3K79-2me should be confirmed by downregulating DOT1L, the actual methyltransferase.
3- In Figure 4 and 5 the co-immunoprecipitation experiments were performed with rabbit polyclonal antibodies, while for the control immunoprecipitations the authors used mouse IgG. The correct control should have been rabbit IgG.
4- In Figure 4 the authors only show H3K79-2me, they should also show total H3; the exhibit in Panel B is not really convincing, do they have any better exhibit? The IP was performed with anti-H3 antibodies, so the conclusion has to be that Bat3 and DOT1L bind histone H3. Binding to H3K73-2me, as the authors state, cannot be assessed from this experiment. An immunoprecipitation with H3K79-2me is required to support the statement. Detection of H3K79-2me by western blotting after immunoprecipitating total histone H3 is expected and does not prove anything.
5- Figure 5 is very confusing. In Panel B the authors present a co-immunoprecipitation where they show that the UBL domain of Bat3 is important for the interaction of Bat3 with DOT1L. While the Figure clearly states that Bat3 was the protein immunoprecipitated, the text on page 13 refers to an
H3 immunoprecipitation. Further down in the same page the text keeps discussing the interaction between DOT1L and H3, supposedly shown in Figure 5B. Either the Figure or the text has to be wrong and the situation needs to be clarified because the conclusions drawn may be different.

6- For the experiment shown in Figure 6 the authors substitute a UIM domain of DOT1L with three repeats of the NAAIRS sequence and in the text they stress that such sequence does not affect the protein. How can they say this? I don't think they can base their work on the fact that NAAIRS sequences were reported in 1985 not to affect different proteins. Ideally the should show that NAAIRS repeats do not affect DOT1L, for example testing its methyltrasferase activity, if the authors do not intend to resent this control, they should at least reduce the emphasis on the fact that the DOT1L UIM protein is otherwise like a wt and warn the reader that their results could also be explained if the DOT1 UIM version is like a complete loss of function.

Does Bat3 influence also mono- and tri- methylation?

Minor comments
1- I don't think the authors can state that after Bat3 knockdown there is a reduction in the size of 53BP1 foci, not form the exhibit in Figure 1 at least.
2- Since Bat3 has been reported to affect gene expression (Nguyen 2008), it would be useful to show in Figure 1 that the level of DOT1L and 53BP1 are not affected by downregulation of BAT3
3- in Figure 2A the authors should indicate directly in the Figure what are the samples analyzed.
4- in Figure 2C the H3pS10 western in shBat3 cells is completely different form its SC control. Please explain.
5- In Figure S4 there is something wrong with the FACS profiles in HeLa SC cells, please check them.
6- Please explain, in the legend to Figure 4, what the different colors correspond to. In general I find the legends not very explicative.
7- Please explain in the legend to Figure 4 how the quantification/normalization was obtained.
8- on page 12 the authors mention a decrease in survival of shBat3 cells and refer to Figure S3. This data are not present in S3, they probably mean S5, which is not mentioned anywhere in the manuscript.
9- on page 17 line 16, the authors talk about H4K20 levels, they must mean H4K20-2me levels. Please correct. Moreover, in the same sentence I don't agree that their results show that when H4K20-2me levels are low, H3K79-2me is required for 53BP1 foci formation, this is why I believe that the control requested in major comment point 2 above is crucial.
10- On page 20 lines 13 and 14, the authors refer to different Bat3 constructs and mention that they were described previously, but fail to cite where they were described. Moreover they refer to a NLS construct that is not mentioned elsewhere in the manuscript. Finally, the inclusion of a minimal description of the structure of the UBL mutant would be useful.
Thank you very much for giving us the opportunity to revise our manuscript entitled “Bat3 Facilitates H3 K79 Dimethylation by DOT1L and Promotes DNA Damage-induced 53BP1 Foci at G1/ G2 Cell Cycle Phases”. We sincerely appreciate all three reviewers for their critical reading of the manuscript and their constructive and insightful suggestions that are truly helpful to clarify and strengthen our conclusion/arguments in the previous version.

In this revised edition, we have addressed all questions from the reviewers with data that are included in the revised manuscript. The text of manuscript has been changed to describe those new data.

We hope that you are satisfied with our revised manuscript and will accept it for publication in EMBO J. Thank you for your consideration, and we are looking forward to hearing from you.

Response to specific comments from reviewers:

Referee #1:

In this manuscript the authors propose that Bat3 facilitates H3K79 dimethylation by DOT1L and recruitment of 53BP1 to DNA damage foci in G1/G2 cells. Overall, this is an interesting manuscript that could become acceptable for publication, if the comments mentioned below can be addressed.

We thank this reviewer for the positive assessment of our work, and the insightful suggestions that have helped us to clarify and broaden our conclusion/arguments with new experimental data.

1. Fig. 1 shows that depletion of Bat3 reduces the average number of 53BP1 foci per cell (Fig. 1A) and the degree of H3K79-dimethylation (Fig. 1B). Fig. 1A is very difficult to visualize; the authors should show the outline of the nuclei and not merge the DAPI staining with the 53BP1 foci. They also should show a bar graph of the percent of cells with more than 5 foci per cell after IR treatment. Their premise is that the average number of foci per cell is decreased, because in some cells (those in G1/G2) 53BP1 does not localize to foci. This would be evident in a bar graph showing the fraction of cells with more than 5 foci per cell after IR treatment.

To address this question, we repeated 53BP1 foci staining in Bat3 knockdown cells and confirmed that knockdown of Bat3 resulted in significantly reduced 53BP1 foci formation following IR treatment. The number of 53BP1 foci per cell and the percentage of 53BP1 foci positive cells were calculated by counting at least 200 cells. As requested, we have now included all the data on DAPI staining to show the outline of the nuclei. The average 53BP1 foci per cell as well as the percentage of foci positive cells are now shown in the form of bar graphs in the revised manuscript (new Fig. 1A).

2. Fig. 2A: which are the scrambled siRNA treated cells and which the Bat3-shRNA treated cells? If the cells express stable-inducible Bat3 shRNA, the authors should show the stably-transfected
cells before and after induction of the Bat3 shRNA. Also as in Fig. 2A, a bar graph of the percent of cells with more than 5 foci per cell after IR treatment should be shown.

We apologize for the unclear marking of the original figure and have now made modifications according to the suggestion. The panel marked SC means scramble control siRNA treated cells and that labeled Bat3 shRNA means Bat3-shRNA treated cells in the revised Fig. 2A. We have also now shown the results of 53BP1 staining in the presence and absence of Doxycycline (for induction of the siRNA) and presented the percentage of 53BP1 foci-positive cells in bar graph as suggested (new Fig. 2A).

3. Fig. 2B: the synchronization of the cells needs improvement. Are the cells grown in the presence of nocodazole? Why is H3pS10 maximum at 20 hours in the shBat3 cells? At that time, synchronized U2OS cells should be in G1. Also why is there H3pS10 staining at all time points in the scrambled shRNA cells? I would like to see this experiment repeated in its entirety. This experiment should also be performed to support the major points of the authors (for example, if WT Bat3, but not Bat3delUBL, rescues 53BP1 focus formation; same for DOT1L WT and delUIM; see below).

Following this suggestion, we have repeated the synchronization experiments in its entirety. We used thymidine (not nocodazole) to synchronize U2OS cells at the G1/S boundary and determined levels of H3pS10 that exhibited only in G2 or G2/M phase during this experiment (new Fig. 2C). This whole experiment was repeated three times and consistent data were obtained to show that H3K79-2Me is at a consistent level throughout the cell cycle in SC cells, whereas it is significantly decreased when Bat3 is downregulated. In contrast, H4K20-2Me is present predominantly at G1/S and S phase and the pattern is not changed when Bat3 was silenced (Fig. 2C). Employing the same system, we have repeated other experiments to show that WT Bat3 but not Bat3 delUBL, as well as wtDOT1L but not DOT1L delUIM, could rescue 53BP1 foci formation in Bat3 knockdown cells (new Fig. 5C and 6D).

4. Fig. 2D: the images should be shown as described above for Fig. 1A.

We have modified the original Fig. 2D and presented it as requested by the reviewer with images of DAPI staining alone and bar graphs showing foci number per cell and percentage of foci-positive cells.

5. Fig. 3B: the images should be shown as described above for Fig. 1A.

We have modified the presentation of Fig. 3B as requested.

6. Fig. 4. Coimmunoprecipitation is not the same thing as colocalization. For colocalization, the authors need to show microscopy images. Figs 4A and 4B are not convincing. Thus, this figure should and the conclusions derived from it should be deleted in its entirety.

We agree that co-immunoprecipitation is not the same as co-localization. We performed co-staining of endogenous 53BP1 and DOT1L and found that these two proteins are constitutively co-localized at nuclear region in U2OS cell. The images are now shown in
We repeated the co-IP experiments for Fig. 4A and obtained better results which exhibit co-IP of Bat3, H3 and DOT1L brought down each of the other proteins including H3K79-2Me (new Fig. 4A). We also repeated H3 IP for Fig. 4B and acquired clearer data showing that knockdown of Bat3 resulted in a marked decrease in the interaction between Bat3 and DOT1L (new Fig. 4B). Since Reviewers #2 and #3 indicated to see the results shown in the original Fig. 4A and 4B, we have decided to present the newly generated and improved data in the revised manuscript.

7. Fig. 5B: the authors should show the level of expression of the wt Bat3 and delUBL Bat3 proteins.

Following the suggestion, we have now included the data (as 4% of Input) in the new Fig. 5B showing the level of expression of the wtBat3 and Bat3delUBL proteins in the Bat3 knockdown cells (they are comparable to that of the endogenous protein marked as SC).

8. Fig. 5C: the images should be shown as described above for Fig. 1A.

We modified the presentation of original Fig. 5C as requested with bar graphs in the new Fig. 5C.

9. Fig. 6A: what is NAAIRS, and NAAIRS x 3? The consensus sequence of the UIM motif should be shown, as well as the UIM sequences of a couple of crystallized motifs showing also which residues interact with ubiquitin.

We apologize for not providing a clear description of the NAAIRS substitution sequence. As referenced in the manuscript with the original paper published in 1985, this strategy has been widely used to generate mutant forms of proteins without the high possibility of disrupting overall protein folding. Several laboratories in our department and our collaborators have used this strategy in the years, and we have attached below a partial list of publications from them since it would be difficulty to cite a large number of papers for this purpose in the manuscript.


Thomas E. Meigs‡§, Juhi Juneja‡, C. Todd DeMarco, Laura N. Stemmler, Daniel D. Kaplan, and Patrick J.


For UIM on DOT1L, we have modified Fig. 6A to illustrate the conserved sequences and specific residues that interact with ubiquitin based on the crystal structure of several proteins (a recent report on this specific topic is also cited in the revised manuscript for this purpose).

10. Does addback of DOT1LdelUIM rescue 53BP1 foci after IR treatment? (Fig. 6B).

To address this question, we conducted the suggested add-back experiment and new data are now shown in the new Fig. 6C and 6D. The results showed that DOT1L knockdown leads to a marked decrease in H3K79-2Me and 53BP1 foci formation, and add-back of wtDOT1L can fully rescue these defects. However, DOT1L delUIM fails to rescue the phenotype, suggesting that the UIM domain of DOT1L is required for DOT1L-mediated regulation of 53BP1 foci formation after IR treatment.

Referee #2:

DOT1L methylates H3K79. While one initial study suggested a role for DOT1L in formation of DNA damage induced 53BP1 foci, several more recent studies have not been able to confirm this result. In fact, most studies suggest that 53BP1 is recruited by H4K20me2. Bat3 has been linked to DNA damage checkpoints and has been shown to affect H3K4 methylation. Here the authors investigate the possible links between DOT1L and Bat3 and their role in formation of DNA damage induced 53BP1 foci.

The authors first confirm that Bat3 is required for DNA damage induced 53BP1 foci formation. The effect of Bat3 knock-down is low in G1/S arrested cells but greater after release of the cells into the cell cycle, when the levels of H4K20me2 start to drop. This leads the authors to suggest that not H4K20me2 but some other mark may be involved in 53BP1 recruitment. The authors suggest that this could be H3K79me2 (e.g. see abstract). However, no direct evidence is provided.

Data presented in the manuscript suggest that Bat3 and DOT1L bind to each other via a ubiquitin-like interaction. The binding of Bat3 to DOT1L by co-immunoprecipitation is lost upon
deletion of the newly identified Ub-interaction motif of DOT1L or upon deletion of the Ub-like motif of Bat3. Reduced levels of Bat3 or mutations in the interaction motifs lead to lower levels of H3K79me2, indicating that the Bat3-DOT1L interaction is required for the methyltransferase function of DOT1L.

Although the interaction between Bat3 and DOT1L, the effect of Bat3 on H3K79me2, and the role of Bat3 in DNA damage response (DDR) is interesting, it is still unclear how Bat3 affects DOT1L function and whether Bat3 affects the DNA damage response via its interaction with DOT1L. In contrast to several statements in the paper that ‘H3K79-2Me is required for IR-induced 53BP1 foci’, no direct evidence is provided for a role of DOT1L or H3K79me2 in this process. Bat3 affects the DNA damage response and H3K79me2, but that does not prove that Bat3 affects the DNA damage response via H3K79me. This, together with inconsistencies between figures, inaccuracies in the text, and incorrect citation of published literature dampens my enthusiasm for this paper.

We thank this reviewer for his/her sincere criticisms and insightful suggestions. We agree that we should take this opportunity to not only reveal new insights but also clarify some issues that are still under debate in the field. Based on the comments and questions, we have performed a large number of experiments to support our conclusion as well as explain and clarify our arguments.

In this study, our main aim was to investigate the role of Bat3 in regulating DOT1L function in the context of DNA damage response. Since 53BP1 recruitment can be affected by H3K79-2Me mediated by DOT1L (Huyen et al., 2004), we chose 53BP1 foci formation as a marker for DNA damage response. We noticed in the literature that there have been several studies showing that 53BP1 recruitment is influenced by H4K20-2Me, not by H3K79-2Me (Botuyan et al., 2006; FitzGerald et al., 2011). However, the level of H4K20-2Me has been demonstrated to be cell cycle-regulated with its peaking during S phase and declining during G1 and G2/M phase. We hypothesized that H3K79-2Me might play a role in 53BP1 recruitment during G1 and G2M phase since H3K79-2Me has been demonstrated to be unchanged throughout the cell cycle (new Fig. 2C). Our synchronization experiment clearly showed that when Bat3 was down-regulated, H3K79-2Me was significantly decreased throughout the cell cycle without changes in the H4K20-2Me expression pattern. Correspondingly, we found that when H3K79-2Me was low, 53BP1 foci formation was also significantly decreased, suggesting that H3K79-2Me regulated by DOT1L is required for 53BP1 foci formation during G1 and G2/M phase (new Fig. 2C).

DOT1L is the only known methyltransferase for H3K79-2Me, and it has been demonstrated that 53BP1-targeting to the sites of DNA DSB is mediated by its interaction with histone H3 at Lys79 methylated by DOT1L (Huyen et al., 2004). Our add-back experiments of wtDOT1L also clearly indicated that down-regulation of DOT1L led to a marked decrease in H3K79-2Me and reduced 53BP1 foci formation and add-back of wtDOT1L was able to rescue 53BP1 foci formation, suggesting that H3K79-2Me is required for 53BP1 recruitment following IR (new Fig. 6C and 6D). Moreover, Bat3 is required for the efficient binding of DOT1L and H3 (new Fig. 4B), and downregulation of Bat3 resulted in a significant decrease in H3K79-2Me that is supposedly catalyzed by
DOT1L (Fig. 1 and Fig. 2). Together, we propose here that the effect of Bat3 on DNA damage response is most likely relevant to its interaction with H3K79-2Me/DOT1L.

Major comments

1. The claim made in the paper and in the abstract that 'H3K79-2Me is required for IR-induced 53BP1 foci' is not supported by the data. It is inferred from phenotypes of Bat3 knock-downs or Bat3 Ubl-domain mutants. However, Bat3 has several known functions unrelated to DOT1L (such as regulation of H3K4 methylation). Therefore, the Bat3 DDR phenotypes may be unrelated to DOT1L. To investigate the role of DOT1L directly the authors should examine the phenotypes of DOT1L knock-downs or of DOT1L-UIM mutants.

Following the suggestion, we have performed the experiments to directly investigate the role of DOT1L by examining the phenotypes of DOT1L knockdown and the DOT1L-UIM mutant as shown in the new Fig. 6C and 6D, as well as new parts of Fig. 1 and 2. Our findings indicated that downregulation of DOT1L leads to a marked decrease in H3K79-2Me and 53BP1 foci formation. While add-back of wtDOT1L is able to rescue the defect in H3K79-2Me and 53BP1 foci formation, DOT1ldelUIM is not able to do so, suggesting that H3K79-2Me is directly related to 53BP1 recruitment following IR treatment (new Fig. 6C and 6D). These findings are consistent with a previous report by Huyen et al in 2004. Furthermore, we uncovered that Bat3 can interact with DOT1L and is required for the binding of DOT1L to H3 (new Fig. 4A and 4B), presumably because Bat3 could stabilize DOT1L on H3 and promote its methyltransferase activity. In addition, Bat3 knockdown caused a marked decrease in H3K79-2Me and 53BP1 foci formation, which are the same phenotypes as those of DOT1L knockdown (Fig. 1, Fig. 2, Fig. 6C and 6D). Based on these results, we propose that Bat3’s function in DNA damage response is most likely related to its regulation of the activity/recruitment of DOT1L.

2. Throughout the paper many of the statements are inaccurate. I give a few examples:

- Abstract and text: 'K79 dimethylation (H3 K79-2Me) by DOT1L influences the DNA damage response by promoting 53BP1 recruitment to DNA damage sites'. As the authors explain elsewhere, most of the studies on this topic suggest that DOT1L does not play a role in the DDR. I would say that at best the role of DOT1 is still under debate. The authors suggest that the U2OS cells investigated here may be different from HeLa cells studied previously. It would be appropriate to note that the study of Botuyan et al found no effect of DOT1L in several cell lines (DOT1L knock-down in HeLa and A549 adenocarcinoma cells, and primary DOT1L +/- MEFs).

- Discussion 'It is important to note that since over 90% of all H3 is methylated, the observed ~50% reduction in H3K79-2Me suggests that Bat3 regulates histone H3 methylation across a broad spectrum of the genome'. This makes no sense. In yeast ~90% of H3K79 is methylated. In mammals, this is much less, as can be found in several high quality mass spectrometry studies.

We apologize for any perceived inaccurate statements and have done our best to modify/correct them.

To clarify the issue with the results reported by Botuyan et al that DOT1L has no effect on 53BP1 foci formation as shown in HeLa, A549 and DOT1L/-MEF cells, we synchronized U2OS and HeLa cells transfected with SC or siBat3 constructs and tested
these cells for IR-induced 53BP1 foci formation at all phases of the cell cycle. We found that HeLa cells exhibited only a slight reduction in 53BP1 foci formation whereas U2OS cells displayed a noticeable reduction in 53BP1 foci formation when Bat3 levels were suppressed (new Fig. S3), suggesting that the effect of H3K79-2Me, and thus DOT1L on 53BP1 recruitment might indeed be cell type specific. We have clearly stated these results in the revised text to clarify the confusion.

We have deleted this statement in Discussion section --- “It is important to note that since over 90% of all H3 is methylated, the observed ~50% reduction in H3K79-2Me suggests that Bat3 regulates histone H3 methylation across a broad spectrum of the genome”.

3. The mechanism by which Bat3 enhances DOT1L activity remains unknown. The authors could test whether the UBL domain is sufficient for Dot1 activation or whether the large C-terminal part of Bat3 is also involved. This may help to generate separation-of-function mutants. Another experiment that would be very informative is to check whether deletion of the UIM of DOT1L affects the binding of DOT1L to H3 or chromatin.

Following the suggestion, we have performed co-IP experiments to see if deletion of the UBL domain could affect DOT1L activity on H3K79-2Me, and the results clearly indicate that UBL is essential for DOT1L to perform its function, whereas the rest of Bat3 protein is not sufficient for DOT1L activation (new Fig. 5). On the hand, we found that deletion of UIM of DOT1L did not influence the binding of DOT1L to H3 (new Fig. S5C). Thus, DOT1L recruitment and/or stabilization to the chromatin/H3 is most likely mediated by its interaction with Bat3 through the UBL domain.

4. Abstract and text and Fig. 4B: 'Bat3 knockdown results in decreased DOT1L-H3 interaction'. The evidence to support this important claim is not very strong (Fig 4B). The bands are not very strong/straight and the overall IP (H3 signal) seems lower in the siBat3 sample. To substantiate this point, which is very important for the interpretation of the further studies, the authors should show biological replicates. The authors should also consider alternative assays to determine the effect of Bat3 on DOT1L. For example, they could do ChIP to determine DOT1L binding to target loci in a quantitative manner, and they could do immunostaining to determine whether the nuclear localization is affected (as in the CALM-AF10 expressing cells that are mentioned in the paper) in Bat3 mutants or DOT1L-UIM mutants.

We have repeated this experiment several more times and obtained much better and reproducible results that clearly showed that Bat3 knockdown leads to a noticeable reduction in DOT1L-H3 interaction (new Fig. 4B). This result is further supported by the new IF results showing the co-localization of Bat3 and DOT1L (new Fig. 4D).

5. I am concerned about the variation between experiments. For example, the Bat3 IP gives a weak DOT1L co-IP in Fig 4A and a very strong co-IP in Fig. 5.
We have repeated Bat3 co-IP experiments as suggested, and obtained much more consistent results that were used to replace the original panels as shown in the new Fig. 4A and Fig. 5B.

6. Figure 2 raises many questions. Is H4K20me2 higher in shBat3? Do the shBat3 cells not progress to G1 or do they progress slower? The label suggests they stay in G2/M. This can affect several of the phenotypes studied. The plot of Fig. 2D does not seem to match with the images shown on the left. For example, at 5 h the number of foci seems much lower in the siBat3 sample, but this is not the case in the graph.

In response to this comment and a similar concern from Reviewer #1, we repeated the experiments and replaced the old results shown in the original Fig. 2. The new results presented in the new Fig. 2C and 2D clearly indicate that both H4K20-2Me and H3pS10 exhibited a similar pattern in both control (SC) and Bat3 knockdown cells.

7. Figure 3. Also here in panel B the images do not seem to match the graphs. The authors should explain this discrepancy. Why is the exposure of the images different?

To address this concern, we repeated multiple times the experiment with γH2AX foci staining in SC and siBat3 cells, and presented the data in the new Fig. 3B, which strongly support our conclusion.

Other Comments:

8. Most of the figure legends do not contain enough information to understand the figures. For example, the authors need to provide more information on the number of cells/biological replicates used for the plots and they need to explain the error bars. I guess they refer to technical replicates.

Also, it is not clear how the intensities were normalized to generate the bar diagrams.

Following this suggestion, we have revised all figure legends including supplemental figure legends to contain necessary information for the understanding of the figures. In the relevant legends we now describe how many cells were used as biological replicates in each experiment and the meaning of the error bars.

We used Image J software and measured the intensity of band for SC and Bat3 siRNA samples and then normalized the intensity of each band to that of the SC band.

9. Abstract: 'The methyltransferase DOT1L methylates histone H3 at K79, facilitating H3 interaction with various binding partners'. Binding partners of H3K79me have not been identified yet.

In Abstract, we changed this statement into "The methyltransferase DOT1L methylates histone H3 at K79 to facilitate specific transcriptional events".

10. Throughout the paper the authors focus on dimethylated H3K79. From what I understand, it
has not been established which of the methylation states of H3K79 are relevant for DOT1L's functions.

Based on the results of the previous report by Huyen et al, it appears that dimethylation of histone H3 at Lys79 is required for 53BP1 foci formation. Since DOT1L is the only known enzyme that could carry out di-methylation of H3K79, it is likely dimethylation of this residue is most relevant for DOT1L's function due to the observations described in this manuscript on the critical role of DOT1L in DNA damage response,

11. Discussion: 'it has been clearly demonstrated that in budding yeast the 53BP1 homolog Rad9 forms foci by binding to histone H3K79 residues that are dimethylated exclusively by Dot1'. In my opinion, there is no direct evidence for the interaction between Rad9 and methylated H3K79. Also, which of the methylation states is involved is still unknown.

We noticed that there were two published papers (Wysocki et al., 2005; Grenon, Costelloe et al. 2007) showing that in budding yeast the 53BP1 homolog Rad9 interacts with methylated H3 at Lys 79. We have changed the statement into “it has been suggested that in budding yeast the 53BP1 homolog Rad9 binds to H3 at Lys79 methylated by Dot1, the yeast homolog of DOT1L”.

12. The binding of Dot1 to H3 is referred to as co-localization. This can generate confusion because co-localization of chromatin proteins usually refers to co-localization in the same compartment or genomic regions. Perhaps the word binding would be more appropriate.

Following this suggestion, we have changed the expression “Bat3 is required for efficient co-localization of DOT1L to H3” into “Bat3 is required for efficient binding of DOT1L to H3”.

13. Very frequently the authors do not correctly cite the published literature. In addition, published data is sometimes discussed without providing a proper reference. The authors should more accurately and more extensively refer to published work. I give a few examples:

- No citation is given for the repeated statement that 'H3 K79-2Me levels in humans remain consistently high and do not appear to fluctuate throughout the cell cycle'.

We apologize for the inaccuracies and inadequacies in citing published literature in the original manuscript. We have thoroughly gone through the text to remedy this deficiency, including the cited example.

- 'In heterochromatin, these dimethylated residues are thought to play a role in higher-order chromatin structure where the residues may be sequestered within the nucleosomes.' No references are given. I am not aware of studies showing that the residues may be sequestered.

We have added the proper citation in the Introduction, and changed this statement into “In heterochromatin, these dimethylated residues have been hypothesized to map to the histone core and would be inaccessible to 53BP1 if higher-order chromatin structure involves nucleosome stacking.”

- 'correlation between ubiquitination of histone H2B at K120 in humans and K123 in yeast with enhanced H3K79 methylation (Jeltsch and Rathert, 2008; Martini et al., 2002; McGinty et al., 2008)' Is Martini the correct citation here?
We apologize for the mistaken citation here and have deleted Martini et al, 2002 from the revised text.

Results: 'Interestingly, HeLa cells display high levels of H4K20-2Me'. Please provide a reference.

We referred this result as shown in Fig. 2C, but should not comment on the levels of H4K20-2Me simply based on the intensity of the protein band, so we have deleted this statement in the revised text.

Referee # 3:

The manuscript by Wakeman et al. analyzes the mechanisms underlying the recruitment of 53BP1 at DNA damage sites. Ample evidence indicates that histone modifications play a critical role in this process and the basics of the mechanisms appear to be evolutionarily conserved. What is not clear in human cell is whether 53BP1 is recruited by H3K79 dimethylation, by H4K20 dimethylation, or both.

In this manuscript the authors address this question and propose that the both modifications contribute but their individual involvement is cell cycle specific. Furthermore they propose that Bat3, a protein previously known to regulate histone H4 modification on K4, by interacting with histone H3 and the histone methyltransferase DOT1L, modulates H3K79 dimethylation. The subject of the investigation is certainly interesting and in general the manuscript is very well written, the logic of the experiments is very clear. The quality of the exhibits is somewhat variable, being better in the first half of the paper. On the other hand there are several controls that are missing and that should be included to make the paper complete and conclusive.

We thank this reviewer for the positive evaluation of our manuscript and for the thoughtful and insightful suggestions.

1- In Figure 1 the authors show that siBat3 leads to a 50% reduction in H3K79-2me. This is the fundament for the rest of the paper. In the same experiment they should also quantitate the effect of Bat3 downregulation on H3K79 mono- and tri- methylation.

As requested, we examined the state of H3K79 mono- and tri-methylation in the absence of Bat3 by Western blot, and the results showed that H3K79 mono- and tri-methylation were evidently decreased when Bat3 was reduced (new Fig. S1B), further supporting a role for Bat3 in regulation of DOT1L.

2- One of the major conclusions of this work is that H4K20-2me is important for 53BP1 foci formation during S phase, while H3K79-2me is relevant in G1 and G2, when H4K20-2me is very low. This is an interesting interpretation but needs to be supported by more direct evidence.
The experiment shown in Figure 2 is based on the fact that downregulation of Bat3 causes a 50% decrease in H3K79-2me. Since Bat3 is also involved in other processes, the cell cycle dependent contribution of H3K79-2me should be confirmed by downregulating DOT1L, the actual methyltransferase.

Following this suggestion, we performed a similar experiment as that shown in the new Fig. 2C for Bat3 by knocking down DOT1L and found that reduction in DOT1L resulted in a significant decrease in H3K79-2Me throughout the cell cycle (new Fig. S2B), further implicating that Bat3-mediated regulation of H3K79-2Me throughout the cell cycle is likely due to its effect on DOT1L rather than on other processes.

3- In Figure 4 and 5 the co-immunoprecipitation experiments were performed with rabbit polyclonals antibodies, while for the control immunoprecipitations the authors used mouse IgG. The correct control should have been rabbit IgG.

We apologize for the mis-statement/labeling in the original text about the nature of the antibodies, and have changed “mouse IgG” into “rabbit IgG” in Material and Method section. We did use rabbit normal IgG as the IgG control.

4- In Figure 4 the authors only show H3K79-2me, they should also show total H3; the exhibit in Panel B is not really convincing, do they have any better exhibit? The IP was performed with anti-H3 antibodies, so the conclusion has to be that Bat3 and DOT1L bind histone H3. Binding to H3K73-2me, as the authors state, cannot be assessed from this experiment. An immunoprecipitation with H3K79-2me is required to support the statement. Detection of H3K79-2me by western blotting after immunoprecipitating total histone H3 is expected and does not prove anything.

As requested, we have repeated several times the Bat3, H3 and DOT1L co-IP experiments and more convincing results were obtained to replace the original panels. In addition, we have included the data on total histone H3 (new Fig. 4A). We made the conclusion in the text that Bat3 is required for the efficient binding of DOT1L to histone H3, not to H3K79-2Me. Since Bat3 knockdown would lead to a marked decrease in H3K79-2Me, consequently we could not perform the co-IP using H3K79-2Me antibody since the pull-down of H3K79-2Me is not supposed to be equal in the presence and absence of Bat3.

5- Figure 5 is very confusing. In Panel B the authors present a co-immunoprecipitation where they show that the UBL domain of Bat3 is important for the interaction of Bat3 with DOT1L. While the Figure clearly states that Bat3 was the protein immunoprecipitated, the text on page 13 refers to an H3 immunoprecipitation. Further down in the same page the text keeps discussing the interaction between DOT1L and H3, supposedly shown in Figure 5B. Either the Figure or the text has to be wrong and the situation needs to be clarified because the conclusions drawn may be different.
We apologize for the confusion in the original text regarding this figure. The IP experiment shown in the old Fig. 5B was indeed the result of a Bat3 IP, and we have replaced the original data with newly generated and more convincing data (new Fig. 5B). We have also modified the necessary statements in text to clarify that Bat3 UBL domain is required for the interaction between Bat3 and DOT1L.

6- For the experiment shown in Figure 6 the authors substitute a UIM domain of DOT1L with three repeats of the NAAIRS sequence and in the text they stress that such sequence does not affect the protein. How can they say this? I don’t think they can base their work on the fact that NAAIRS sequences were reported in 1985 not to affect different proteins. Ideally they should show that NAAIRS repeats do not affect DOT1L, for example testing its methyltrasferase activity, if the authors do not intend to resent this control, they should at least reduce the emphasis on the fact that the DOT1LΔUIM protein is otherwise like a wt and warn the reader that their results could also be explained if the DOT1LΔUIM version is like a complete loss of function.

We thank the reviewer for this thoughtful and constructive suggestion on the NAAIRS issue since it was also commented by Reviewer #1.

We have reduced the emphasis about the effect of delUIM using substitution with NAAIRS triple repeats on protein function by changing the statement in the Discussion section into “to study the importance of this domain we chose the strategy of replacing it with a triple NAAIRS repeat because this sequence has been postulated to not disrupt overall protein structure”. The DOT1LdelUIM mutant did not affect the binding of DOT1L to H3 which is critical for the DOT1L methylatransferase activity (shown in the new Fig. S5C), indicating that mutation of UIM likely does not influence the overall structure of DOT1L and its enzymatic activity.

Does Bat3 influence also mono- and tri- methylation?

Yes, we have examined the mono-and tri-methylaiton of H3K79 when we silenced Bat3, and found that Bat3 knockdown also led to marked decreases in mono-and tri-methylaiton of H3K79, further indicating that Bat3 is involved in the regulation of DOT1L function (new Fig. S1B).

Minor comments

1- I don’t think the authors can state that after Bat3 knockdown there is a reduction in the size of 53BP1 foci, not form the exhibit in Figure 1 at least.

We have repeated several more times the 53BP1 foci staining experiment when Bat3 was knocked down and obtained reproducible and convincing results. We have presented the average 53BP1 foci number per cell and the percentage of 53BP1 foci-positive cells in the new Fig. 1A.
2- Since Bat3 has been reported to affect gene expression (Nguyen 2008), it would be useful to show in Figure 1 that the level of DOT1L and 53BP1 are not affected by downregulation of BAT3.

We performed Western blot to examine potential changes in DOT1L and 53BP1 levels when Bat3 was reduced, and found that there was no affect on DOT1L and 53BP1 protein levels by downregulation of Bat3 (new Fig. S1B).

3- In Figure 2A the authors should indicate directly in the Figure what are the samples analyzed.

We have marked what samples were analyzed in Fig. 2A.

4- In Figure 2C the H3pS10 western in shBat3 cells is completely different from its SC control. Please explain.

We repeated the H3pS10 western blot experiment and obtained convincing data showing that H3pS10 levels were similar between SC and shBats cells (new Fig. 2C).

5- In Figure S4 there is something wrong with the FACS profiles in HeLa SC cells, please check them.

Since this piece of data is not essential for the study, we have removed this figure from the revised manuscript.

6- Please explain, in the legend to Figure 4, what the different colors correspond to. In general I find the legends not very explicative.

We have modified all the figure legends to make sure enough information was provided.

7- Please explain in the legend to Figure 4 how the quantification/normalization was obtained.

We have repeated the whole experiment and obtained more convincing results now presented in the new Fig. 4.

8- On page 12 the authors mention a decrease in survival of shBat3 cells and refer to Figure S3. This data are not present in S3, they probably mean S5, which is not mentioned anywhere in the manuscript.

We apologize for the mislabeling and missing information about this piece of data. The data of cell survival of shBat3 cells is now presented in Fig. S4A in the revised manuscript.

9- On page 17 line 16, the authors talk about H4K20 levels, they must mean H4K20-2me levels. Please correct. Moreover, in the same sentence I don't agree that their results show that when
H4K20-2me levels are low, H3K79-2me is required for 53BP1 foci formation. Their results show that in those conditions Bat3 is important for 53BP1 foci formation, this is why I believe that the control requested in major comment point 2 above is crucial.

We apologize for the mistake and have corrected it as we did refer to the levels of H4K20-2Me and not H4K20. We agree with the interpretation of this reviewer on the data and have addressed this question with new results shown in the new Fig. 2C and 2D as discussed earlier in our response to Point 2 of the major comment.

10- On page 20 lines 13 and 14, the authors refer to different Bat3 constructs and mention that they were described previously, but fail to cite where they were described. Moreover, they refer to a ΔNLS construct that is not mentioned elsewhere in the manuscript. Finally, the inclusion of a minimal description of the structure of the ΔUBL mutant would be useful.

We apologize for the missing information on the constructs. We obtained the Bat3 WT and Bat3 ΔUBL constructs from Dr. Sally Kornbluth’s lab in our department and they described them in some of their earlier publications (they called Bat3 Scythe at that time). We have added reference to the manuscript. Since we never presented any data on the Bat3 ΔNLS mutant, we have deleted its reference in the section of Material and Method. The UBL motif consists of about 70 amino acids sequence that is shared by many ubiquitin-binding proteins. This motif is deleted in the ΔUBL Bat3 mutant by site-directed mutagenesis which is briefly described in the section of Material and Methods.
Thank you for submitting your revised manuscript for consideration by The EMBO Journal. All three original reviewers have now looked at it once more, and I am happy to inform you that all of them acknowledge that the manuscript has been significantly improved. There are however still a few remaining, comparably minor issues raised by referees 1 and 2, which nevertheless need to be cleaned up before the paper will be ready for acceptance. I am therefore returning the manuscript to you once more with these comments attached, hoping you will be able to return a re-vised final version to us soon. Pending adequate clarification of the remaining concerns, I hope we should then be able to proceed with the ultimate publication of the manuscript.

Yours sincerely,
Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

Referee #1

In this manuscript the authors propose that Bat3 facilitates H3K79 dimethylation by DOT1L and recruitment of 53BP1 to DNA damage foci in G1/G2 cells.

The authors have addressed all my comments. Figs 2C and 2D convincingly show that H4 K20 dimethylation cannot explain recruitment of 53BP1 to foci in cells that are not in S phase, since these cells lack H4 K20 dimethylation. Thus, 53BP1 recruitment to foci depends on H4 K20 methylation in S phase and H3 K79 methylation in G1/G2.

Fig. 5 also nicely shows that the UBL domain of Bat3 is needed for 53BP1 focus formation and Fig. 6 shows that the ubiquitin-binding motif of Dot1 is needed for 53BP1 focus formation. These results support well the premise of the authors that Bat3 and Dot1 interact and that this interaction leads to H3 K79 methylation and 53BP1 recruitment.

Minor point:

The quality of the figures and graphs can be improved a little bit. In the immunofluorescence panels, the Merge images do not add anything and can be removed.

Comment about point 11 of Referee #2.

I do not agree with Referee #2's statement that there is no evidence that Rad9 (53BP1) in budding yeast binds to methylated H3 K79. Rad9 has been shown to bind methylated H3 K79 in vitro (Huyen et al, 2004) and the analysis of Rad9 deletion, Dot1 deletion and H3 K79A substitution mutants by Muzzi-Falconi (Giannattasio et al., 2005; Lazzaro et al., 2008) prove almost beyond doubt that Rad9 binds to methylated H3 K79. It is also interesting to know that in budding yeast there is no H4 K20 dimethylation and no corresponding methyltransferase. In contrast, in fission yeast, there is no Dot1 homolog and no H3 K79 methylation, but H4 K20 is methylated. This explains why 53BP1 in budding yeast binds to methylated H3 K79, whereas in fission yeast 53BP1 binds to methylated H4 K20. In mammals, which have both H3 K79 and H4 K20 methylation, it is not surprising that 53BP1 can bind to both methylated marks, as the authors of this manuscript demonstrate.
Referee #2 (Remarks to the Author):

The manuscript entitled "Bat3 Facilitates H3 K79 Dimethylation by DOT1L and Promotes DNA Damage-induced 53BP1 Foci at G1/G2 Cell Cycle Phases" from Wakeman et al, reveals connections between Bat3, DOT1L/H3K79 dimethylation, and DNA damage response. The manuscript has much improved. Most of my main initial concerns have been addressed. Many of the inaccuracies in the text and figures were corrected. The authors also performed a number of additional experiments and repeated some of the previous ones to address the main points. In particular, the role of DOT1L in 53BP1 foci formation and gamma H2AX induction has been clarified. Not only have the authors performed critical DOT1L knock-down experiments, they also explain more clearly why they believe that in other cells used for several previous studies, no reproducible role of DOT1L in the DDR could be identified. The results shown strongly suggest that Bat3 is a new DOT1L-interacting protein that regulates the activity of this enzyme. However, I still have a few points and concerns.

1. Upon siBat3 treatment of U2OS cells, a reduction in 53BP1 foci formation is observed. However, in many of the figures (for example Fig 1A), the images with reduced 53BP1 foci formation also show the lowest DAPI staining. To allow a fair comparison and make the results more convincing, it is important that images are shown with equal intensities.

2. The authors show that Bat3 and DOT1L interact through specific domains (Fig 5B and 6B). In addition, Bat3 is required for DOT1L binding to H3 (Fig 4B). The hypothesis is that Bat3 binds DOT1L and recruits it to chromatin to stimulate H3K79-2me and, upon DNA damage, facilitate 53BP1 foci formation. However, not all data support this hypothesis. Fig S5 shows that DOT1L with or without its IUM domain binds H3 equally well. This suggests that while the binding of DOT1L to chromatin depends on Bat3, it does not depend on the interaction between the two proteins. The authors need to explain this and adjust the conclusions in the abstract and main text, and they should consider transferring Fig S5A and C to the main figures. Possibly, the interaction between Bat3 and DOT1L enhances the catalytic activity of DOT1L, since DOT1L IUM has decreased H3K79-2me levels (Fig 6C). Bat3 mediated localization of DOT1L may occur through another mechanism.

3. In Fig 4A in the Bat3 co-IP, the H3 co-IP signal is clearly more abundant than the input signal, while the H3K79-2me co-IP signal is lower than the input signal. This result suggests that Bat3 has a preference for unmethylated H3. These data contradict the statement in the abstract that "Bat3 co-localizes with DOT1L at H3K79-2me...". Furthermore, it is counter intuitive that the IP of a relatively low abundance protein (Bat3/DOT1L) results in significant enrichment for histone H3, a very abundant protein. Was the IP done with soluble histones only? Which percentage of the input was loaded on the gel?

4. It is unclear to me which siRNAs have been used to target Bat3 and DOT1L. In addition, the authors should mention how many and which other siRNAs have been tested for each protein to exclude off-target effects. In addition, information on the H3K79 mono- and trimethyl antibodies is missing.

5. From Fig 4D, it is hard to conclude that the two proteins co-localize. There is a lot of signal throughout the nucleus (and no antibody controls) and a significant proportion of the signals do not seem to overlap. Furthermore, localization of Bat3 seems rather different in Fig S5B. Perhaps siDOT1L and siBat3 controls could be of help here.

6. Given the controversy in the field regarding the role of DOT1L in 53BP1 foci formation, it should be clearly stated in the abstract and the main text that the data were obtained with U2OS cells (since the effects seem to be very much cell type specific).

Minor points:
The y-axis of the graphs in Fig 1A, 1B and 2A and the x-axis of the graph in Fig 3C is not labeled.

Quantification of Fig 1B should show standard deviations, or should not be shown. The legend does
not explain clearly how the bands were normalized.

In the alignment in Fig 6A please add aa residue numbers and explain the NAAIRS sequence in the legend.

It is not clear whether the cells in Fig S3 were irradiated or not. The legend states that they were irradiated while the title states "in the absence of DNA damage".

Page 10: "... depletion of DOT1L resulted in a remarkable decrease in H3K79m-2Me throughout the cell cycle (Fig S2B), indicating that the effect of Bat3 on H3K79-2Me is most likely relevant to the function of DOT1L". I do not understand this sentence and significance of this result. It is not unexpected that depletion of the only known H3K79 methyltransferase (DOT1L) results in a decrease of H3K79-2me.

Referee #3 (Remarks to the Author):

I believe that the authors put a lot of effort in these revisions and greatly improved the manuscript. They answered to the Referees' comments satisfactorily and I am happy with this new version of the paper.
Thank you very much for giving us one more opportunity to revise our manuscript entitled “Bat3 Facilitates H3 K79 Dimethylation by DOT1L and Promotes DNA Damage-induced 53BP1 Foci at G1/ G2 Cell Cycle Phases”. We sincerely appreciate all three reviewers for their critical reading of the revised manuscript and their favorable and insightful comments and suggestions that are truly helpful to further strengthen our conclusion/arguments in the previous version.

In this second revised edition, we have addressed and explained all questions from Reviewer #1 and #2. We hope that you are satisfied with this newly revised manuscript and accept it for publication in *EMBO J*.

Response to specific comments from reviewers:

**Referee #1:**

In this manuscript the authors propose that Bat3 facilitates H3K79 dimethylation by DOT1L and recruitment of 53BP1 to DNA damage foci in G1/G2 cells. The authors have addressed all my comments. Figs 2C and 2D convincingly show that H4 K20 dimethylation cannot explain recruitment of 53BP1 to foci in cells that are not in S phase, since these cells lack H4 K20 dimethylation. Thus, 53BP1 recruitment to foci depends on H4 K20 methylation in S phase and H3 K79 methylation in G1/G2. Fig. 5 also nicely shows that the UBL domain of Bat3 is needed for 53BP1 focus formation and Fig. 6 shows that the ubiquitin-binding motif of Dot1 is needed for 53BP1 focus formation. These results support well the premise of the authors that Bat3 and Dot1 interact and that this interaction leads to H3 K79 methylation and 53BP1 recruitment.

Minor point:
The quality of the figures and graphs can be improved a little bit. In the immunofluorescence panels, the Merge images do not add anything and can be removed.

We sincerely thank this reviewer for the favorable evaluation of our work. As requested, we have removed the merge images from all immunofluorescence panels (Fig. 1A, 2A, 2D, 3B, 3C, 5C, 6D).

Comment about point 11 of Referee #2.

I do not agree with Referee #2’s statement that there is no evidence that Rad9 (53BP1) in budding yeast binds to methylated H3 K79. Rad9 has been shown to bind methylated H3 K79 in vitro (Huyen et al, 2004) and the analysis of Rad9 deletion, Dot1 deletion and H3 K79A
substitution mutants by Muzi-Falconi (Giannattasio et al., 2005; Lazzaro et al., 2008) prove almost beyond doubt that Rad9 binds to methylated H3 K79. It is also interesting to know that in budding yeast there is no H4 K20 dimethylation and no corresponding methyltransferase. In contrast, in fission yeast, there is no Dot1 homolog and no H3 K79 methylation, but H4 K20 is methylated. This explains why 53BP1 in budding yeast binds to methylated H3 K79, whereas in fission yeast 53BP1 binds to methylated H4 K20. In mammals, which have both H3 K79 and H4 K20 methylation, it is not surprising that 53BP1 can bind to both methylated marks, as the authors of this manuscript demonstrate.

We really appreciate this reviewer for helping us to clarify that in budding and fission yeast, 53BP1 binds to methylated H3K79 and methylated H4K20, respectively, and that in mammals, 53BP1 can bind to both methylated H3K79 and H4K20.

Referee #2:

The manuscript entitled "Bat3 Facilitates H3 K79 Dimethylation by DOT1L and Promotes DNA Damage-induced 53BP1 Foci at G1/G2 Cell Cycle Phases" from Wakeman et al, reveals connections between Bat3, DOT1L/H3K79 dimethylation, and DNA damage response. The manuscript has much improved. Most of my main initial concerns have been addressed. Many of the inaccuracies in the text and figures were corrected. The authors also performed a number of additional experiments and repeated some of the previous ones to address the main points. In particular, the role of DOT1L in 53BP1 foci formation and gamma H2AX induction has been clarified. Not only have the authors performed critical DOT1L knock-down experiments, they also explain more clearly why they believe that in other cells used for several previous studies, no reproducible role of DOT1L in the DDR could be identified. The results shown strongly suggest that Bat3 is a new DOT1L-interacting protein that regulates the activity of this enzyme. However, I still have a few points and concerns.

We sincerely thank this reviewer for the positive comments on that the connection between Bat3, DOT1L/H3K79 dimethylation and DNA damage response has been more clearly established in our revised manuscript.

1. Upon siBat3 treatment of U2OS cells, a reduction in 53BP1 foci formation is observed. However, in many of the figures (for example Fig 1A), the images with reduced 53BP1 foci formation also show the lowest DAPI staining. To allow a fair comparison and make the results more convincing, it is important that images are shown with equal intensities.

To address this concern, we have replaced the panels in question with those that show equal intensities of DAPI staining in Fig.1A, Fig.2A and Fig.6D.
2. The authors show that Bat3 and DOT1L interact through specific domains (Fig 5B and 6B). In addition, Bat3 is required for DOT1L binding to H3 (Fig 4B). The hypothesis is that Bat3 binds DOT1L and recruits it to chromatin to stimulate H3K79-2me and, upon DNA damage, facilitate 53BP1 foci formation. However, not all data support this hypothesis. Fig S5 shows that DOT1L with or without its IUM domain binds H3 equally well. This suggests that while the binding of DOT1L to chromatin depends on Bat3, it does not depend on the interaction between the two proteins. The authors need to explain this and adjust the conclusions in the abstract and main text, and they should consider transferring Fig S5A and C to the main figures. Possibly, the interaction between Bat3 and DOT1L enhances the catalytic activity of DOT1L, since DOT1LΔIUM has decreased H3K79-2me levels (Fig 6C). Bat3 mediated localization of DOT1L may occur through another mechanism.

As shown in Fig. 4A, Bat3, DOT1L and H3 can interact each other, and our points are that Bat3 interacts with DOT1L which most likely enhances the enzymatic activity of DOT1L and meanwhile facilitates DOT1L recruitment to chromatin to stimulate H3K79 dimethylation. We discovered that that UIM domain in DOT1L is critical for interaction with Bat3 and for the regulation of DOT1L on H3K79 dimethylation and 53BP1 foci formation upon DNA damage (Fig. 6B, C and D). It is most likely that H3 binds to DOT1L on another domain instead of UIM and that DOT1L ΔUIM mutant can normally bind to H3, but the enzymatic activity of DOT1L ΔUIM mutant decreases due to the reduced interaction with Bat3.

3. In Fig 4A in the Bat3 co-IP, the H3 co-IP signal is clearly more abundant than the input signal, while the H3K79-2me co-IP signal is lower than the input signal. This result suggests that Bat3 has a preference for unmethylated H3. These data contradict the statement in the abstract that "Bat3 co-localizes with DOT1L at H3K79-2me...". Furthermore, it is counter intuitive that the IP of a relatively low abundance protein (Bat3/DOT1L) results in significant enrichment for histone H3, a very abundant protein. Was the IP done with soluble histones only? Which percentage of the input was loaded on the gel?

We did observe a large amount of H3 pull-downed by Bat3 antibody which is more abundant than the input signal presented in the figure. We think that this is because the anti-Bat3 antibody generated from Dr. Sally Kornbluth’s lab was a very good one with high affinity. In contrast, the H3K79-2me co-IP is lower than the input signal because the H3 total antibody is much better than the H3K79-2me antibody. The DOT1L antibody that we purchased from Bethyl Laboratories is also very good for IP and resulted in obvious enrichment for H3. We have revised the abstract and changed the statement into "Bat3 co-localizes with DOT1L at Histone H3......". We used whole cell lysate for IP and the input loaded on the gel was 4% (now described in legend).
4. It is unclear to me which siRNAs have been used to target Bat3 and DOT1L. In addition, the authors should mention how many and which other siRNAs have been tested for each protein to exclude off-target effects. In addition, information on the H3K79 mono- and trimethyl antibodies is missing.

We used the exactly the same siRNAs described in published reports (Bat3 siRNA from Sasaki et al. 2007; and DOT1L siRNA from Huyen et al. 2004; now cited in Material and Methods). In this study, we also used a Bat3 shRNA inducible system and found similar effects on 53BP1 foci formation upon DNA damage (Fig.1A and Fig.2A), confirming the results from the use of siRNAs. The target sequences of Bat3 siRNA and shRNA are different, thus the off-target effects can be excluded. We purchased the H3K79 mono- and tri-methyl antibodies from Abcam and have described them in Material and Methods.

5. From Fig 4D, it is hard to conclude that the two proteins co-localize. There is a lot of signal throughout the nucleus (and no antibody controls) and a significant proportion of the signals do not seem to overlap. Furthermore, localization of Bat3 seems rather different in Fig S5B. Perhaps siDOT1L and siBat3 controls could be of help here.

We actually found that Bat3 is localized at both nucleus and cytoplasm and that DOT1L is mainly localized in the nucleus. We did observe that there is a significant co-localization between these two proteins in the nucleus (see the yellow signals) and that if either Bat3 was knocked down or DOT1L was knocked down (following the suggestion by this reviewer), the co-localization was remarkably decreased (new Fig.4D).

6. Given the controversy in the field regarding the role of DOT1L in 53BP1 foci formation, it should be clearly stated in the abstract and the main text that the data were obtained with U2OS cells (since the effects seem to be very much cell type specific).

We have stated that the connection of DOT1L with 53BP1 foci formation regulated by Bat3 was observed in the U2OS cells in the Abstract and in the main text wherever is necessary.

Minor points:
The y-axis of the graphs in Fig 1A, 1B and 2A and the x-axis of the graph in Fig 3C is not labeled.

We have labeled the y-axis of graphs in Fig.1B and 2A and the x-axis of the graph in Fig.3C.
Quantification of Fig 1B should show standard deviations, or should not be shown. The legend does not explain clearly how the bands were normalized.

Although the experiments were done three times, the quantification of Fig.1B was performed for the experimental results shown, so there is no standard deviation for the results. We have explained how the bands were normalized in the legend.

In the alignment in Fig6A please add aa residue numbers and explain the NAAIRS sequence in the legend.

We have now mentioned that the residues Ala365, Ser374 and Ala376 are important for direct interaction with ubiquitin in the legend of Fig.6A.

It is not clear whether the cells in Fig S3 were irradiated or not. The legend states that they were irradiated while the title states 'in the absence of DNA damage'.

We apologize for the inaccurate statement and have changed the title into “in the presence of DNA damage” in the legend of Fig.S3.

Page 10: ".... depletion of DOT1L resulted in a remarkable decrease in H3K79m-2Me throughout the cell cycle (Fig S2B), indicating that the effect of Bat3 on H3K79-2Me is most likely relevant to the function of DOT1L". I do not understand this sentence and significance of this result. It is not unexpected that depletion of the only known H3K79 methyltransferase (DOT1L) results in a decrease of H3K79-2me.

We have rephrased the statement into “Consistent with previously reported findings that depletion of DOT1L resulted in a remarkable decrease in H3K79-2Me throughout the cell cycle (Figure S2B), the observed effect of Bat3 (Figure 2C) on H3K79-2Me is most likely relevant to the function of DOT1L as both Bat3 knockdown and DOT1L knockdown display a similar phenotype in terms of H3K79-2me.”

Thank you for your consideration, and we are looking forward to hearing from you.

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Thank you for submitting your revised manuscript for our consideration. I have now had a chance to look through it and to assess your responses to the comments raised by the original reviewers, and I am happy to inform you that there are no further objections towards publication in The EMBO Journal.

You shall receive a formal letter of acceptance shortly.

Yours sincerely,

Editor
The EMBO Journal