EMBO reports

Manuscript EMBOR-2014-39434

SET8 methyltransferase activity during the DNA double strand break response is required for recruitment of 53BP1

Stanimir Dulev, Johnny Tkach, Sichun Lin and Nizar Batada

Corresponding author: Nizar Batada, Ontario Institute of Cancer Research

Review timeline:

Submission date:	11 August 2014
Editorial Decision:	30 August 2014
Revision received:	04 September 2014
Accepted:	05 September 2014

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.)

Editor: Barbara Pauly

1st Editorial Decision 30 August 2014

Many thanks for your patience while we were waiting to hear back from the reviewers on the suitability of your study for EMBO reports. We have now received the two enclosed reports on it and I am happy to tell you that we can offer publication of your study in our journal after minor revision along the lines of the reviewers' suggestions.

Referee 1 only raises some minor concerns that can be addressed in writing. Referee 3 also raises some minor issues with regard to figures 1 and 2. With regard to his/her point about the synchronized vs. non-synchronized cells, we feel it would be sufficient to clearly state the fact that the cells were not synchronized and to tone down the claim that 53BP1 recruitment is independent of PCNA. Of course, if you already have obtained data on synchronized cells, we encourage you to include those, but would not make this a prerequisite for publication. With regard to the fact that inhibition of HDACs leads to enhanced SET8 recruitment to DSBs, while at the same time not leading to an accumulation of 53BP1, we also feel that it would be sufficient to more clearly discuss this apparent contradiction, rather than performing the experiments suggested by this reviewer. But again, if you already have data along those lines, I would encourage you to include them.

In summary, we feel confident that you will be able to address these minor changes in a rather timely fashion so that the publication of the manuscript is not delayed any further. As soon as you have submitted the final version I will make sure that it gets fast-tracked for publication.

I look forward to seeing a revised form of your manuscript when it is ready. Should you in the meantime have any questions, please do not hesitate to contact me.

REFEREE REPORTS:

Referee 1:

In this paper the authors have addressed the role of the Set8 protein in recruitment of 53BP1 to DNA damage sites. The experiments are well done and in general support the conclusions made. Their findings are also consistent with Tuzon et al's recent publication. Two notable advances in this current manuscript are the authors' use of the auxin degradation system to demonstrate that 53bp1 recruitment to DDR sites is markedly reduced within 6 hours of Set8 loss, thus ruling out a possible side-effect of prolonged reduction in global H4K20me1 levels. In addition, the authors show that Set8 plays a role an important role in NHEJ and its recruitment to DDR sites can occur independently of its interaction with PCNA. This latter point brings up the issue of how Set8 degradation in S phase in a PCNA dependent manner might interfere with or regulate its role in the DNA damage response. A few minor changes to improve the quality of the manuscript are listed below:

Minor points

1. In Figure 1G, the legend is not clear. One can infer that the first three lanes are sirna to control and the last three sirna to Set8. However this should be clearly stated.

2. In Figure 3B, some quantification of the localization of set8 mutants would be helpful.

3. In the discussion section on Page 6, should lines 1 and 2 read "negatively regulated by HDAC inhibition" and not "by HDACs"?

4. The lack of a GFP signal in the NHEJ assay does not mean NHEJ is defective. It could mean that the rate of excision is changed such that when the NHEJ does eventually occur, it no longer generates GFP. Thus it could alter NHEJ as opposed to be required for it.

Referee 3:

General comments: The manuscript from Dulev et al deals with the role of the SET8 Histone H4K20 monomethyltransferase in the DNA double strand break response (DDR), particularly linked with the recruitment of the repair factor 53BP1. This is a topic that has been quite extensively investigated in the past. The role of SET8 in 53BP1 accumulation at damage sites has now become reasonably established through the published work from the Reinberg (PMID: 21035370) and Scully labs (PMID: 23209566) among others. Recently, the Rice lab identified a more direct DDR link between SET8 and 53BP1 recruitment involving de novo methylation. Furthermore, the Rice lab also described a role for the NHEJ repair factor Ku70 as a DDR recruitment factor for SET8 (PMID: 25001286).

The data in the manuscript from Dulev et al confirm and are generally consistent with the previous work outlined above, including the recent paper from the Rice lab. Obviously, there is an issue with the novelty of the current manuscript from Dulev et al., and it appears beneficial for the authors to develop their manuscript further. First, Dulev et al have generated an intriguing SET8 degron system that could be used for further investigations of SET8 function. In addition, they suggest a role of HDACs in the recruitment of SET8 to break sites. The authors show that treatment of cells with HDAC inhibitors is followed by a strong accumulation of SET8 and H4K20me1 at break sites, suggesting that HDACs limit SET8 recruitment. On the other hand they found that 53BP1 is not recruited to DSBs under these conditions. Although this is an interesting finding, the authors do not elaborate further on this. Why is 53BP1 not recruited, given the positive impact of SET8/H4K20me1 on 53BP1 recruitment? Is H4K20me1 globally increased independent of damage

after HDAC inhibition?

Specific comments:
Figure 1:
Were the expression levels of SET8 wt and R265G mutant carefully controlled?
Fig. 1B: Duration of depletion should be indicated.
Fig. 1F: gamma-H2Ax should be included as a control to show that DDR is active in IR treated SET8-kd cells shown. Duration of depletion should be indicated.
Fig. 1G: siCON and siSET8 should be indicated.

Figure 2:
Fig. 2B: gammaH2Ax should be included as a control to show that DDR is active in IR treated SET8-kd cells shown.
Fig. 2C: H4K20me and actin blots should be improved.

Figure 3:
The authors claim to show that SET8's role in 53BP1 recruitment does not require PCNA, this may be an over simplification. If analyzing synchronized cells, the SET8 dependent recruitment of 53BP1 after IR could be dependent upon PCNA (the phase where PCNA is most active) while this may not be the case in G1 phase.

Figure 4:
The authors show circumstantial evidence of HDACs counteracting SET8 recruitment at DSBs. This part should be markedly expanded. The authors can for example show which HDACs are responsible and nature of interaction (direct or indirect). There is no mention of which concentrations of TSA or Na-Butyrate was used.

Fig. 4E and G: SET8 and H4K20me1 abundance could be analyzed after TSA to strengthen these data.

1st Revision - authors' response 04 September 2014

We want to give our sincere thanks to the Reviewers for taking the time to review our manuscript and for providing helpful feedback to improve it further.

Referee 1:

In this paper the authors have addressed the role of the Set8 protein in recruitment of 53BP1 to DNA damage sites. The experiments are well done and in general support the conclusions made. Their findings are also consistent with Tuzon et al's recent publication. Two notable advances in this current manuscript are the authors' use of the auxin degradation system to demonstrate that 53bp1 recruitment to DDR sites is markedly reduced within 6 hours of Set8 loss, thus ruling out a possible side-effect of prolonged reduction in global H4K20me1 levels. In addition, the authors show that Set8 plays a role an important role in NHEJ and its recruitment to DDR sites can occur independently of its interaction with PCNA. This latter point brings up the issue of how Set8 degradation in S phase in a PCNA dependent manner might interfere with or regulate its role in the DNA damage response. A few minor changes to improve the quality of the manuscript are listed below:

Minor points

1. In Figure 1G, the legend is not clear. One can infer that the first

three lanes are sirna to control and the last three sirna to Set8. However this should be clearly stated.

Figure has been updated and the lanes corresponding to siCON and siSET8 have been marked.

2. In Figure 3B, some quantification of the localization of set8 mutants would be helpful.

We have provided more images in Supplementary Figure 2 showing many laser positive and GFP positive cells and the accumulation of the N-terminal fragment of SET8 and the failure of C-terminal fragment of SET8 to accumulate at laser microirradiation sites.

3. In the discussion section on Page 6, should lines 1 and 2 read "negatively regulated by HDAC inhibition" and not "by HDACs"?

We have corrected the wording.

4. The lack of a GFP signal in the NHEJ assay does not mean NHEJ is defective. It could mean that the rate of excision is changed such that when the NHEJ does eventually occur, it no longer generates GFP. Thus it could alter NHEJ as opposed to be required for it.

We have toned down our wording in the manuscript. In particular, instead of saying SET8 depletion compromised NHEJ we instead say, SET8 depletion altered NHEJ's fidelity or efficiency or progression.

Referee 3:

General comments: The manuscript from Dulev et al deals with the role of the SET8 Histone H4K20 monomethyltransferase in the DNA double strand break response (DDR), particularly linked with the recruitment of the repair factor 53BP1. This is a topic that has been quite extensively investigated in the past. The role of SET8 in 53BP1 accumulation at damage sites has now become reasonably established through the published work from the Reinberg (PMID: 21035370) and Scully labs (PMID: 23209566) among others. Recently, the Rice lab identified a more direct DDR link between SET8 and 53BP1 recruitment involving de novo methylation. Furthermore, the Rice lab also described a role for the NHEJ repair factor Ku70 as a DDR recruitment factor for SET8 (PMID: 25001286).

The data in the manuscript from Dulev et al confirm and are generally consistent with the previous work outlined above, including the recent paper from the Rice lab. Obviously, there is an issue with the novelty of the current manuscript from Dulev et al., and it appears beneficial for the authors to develop their manuscript further. First, Dulev et al have generated an intriguing SET8 degron system that could be used for further investigations of SET8 function. In addition, they suggest a role of HDACs in the recruitment of SET8 to break sites. The authors show that treatment of cells with HDAC inhibitors is followed by a strong accumulation of SET8 and H4K20me1 at break sites, suggesting that HDACs limit SET8 recruitment. On the other hand they found that 53BP1 is not recruited to DSBs under these conditions. Although this is an interesting finding, the authors do not elaborate further on this. Why is 53BP1 not recruited, given the positive impact of SET8/H4K20me1 on 53BP1 recruitment? Is H4K20me1 globally increased independent of damage after HDAC inhibition?

Although H4K20me1 is specifically increased at DSBs but not generally on bulk chromatin

(see Figures 4F and G -- at control sites Chr1_N and Chr2_N are not cut by AsiSI and at these sites H4K20me1 does not go up in HDAC and tamoxifen treated cells), suggesting that although H4K20me1 is necessary it is insufficient for 53BP1 binding to chromatin. Previous studies have shown that H4K16ac increases upon HDAC treatment and also that H4K16ac affects 53BP1 binding. Thus, the simplest explanation of our findings is increase in H4K16ac is in HDACi treated cells is affecting 53BP1's binding to chromatin. This is discussed in the 3rd paragraph of the Discussion.

Specific comments:
Figure 1:
Were the expression levels of SET8 wt and R265G mutant carefully controlled?

The quantification shown in Figure 1G only includes GFP positive cells. We used transient transfection, which does not allow precise control over expression in different samples, but during quantification of images we excluded the cells that had no/faint GFP as well as those that had very high levels of GFP.

Fig. 1B: Duration of depletion should be indicated.

Cells were depleted for 48 hours using 2 subsequent transfections of siCON or siSET8 siRNAs. This information is now included in the figure legends.

Fig. 1F: gamma-H2Ax should be included as a control to show that DDR is active in IR treated SET8-kd cells shown. Duration of depletion should be indicated.

In this specific experiment we did not stain for γH2AX; however, in parallel we did stain for gH2AX in siCON and siSET8 cells and we have included their images in the Supplementary Figure 1. This experiment along with the ChIP experiments (Figure 1C) confirms that the extent of DSBs (as represented by γH2AX) is not affected upon SET8 depletion. Figure legends for Figure 1F has been updated.

Fig. 1G: siCON and siSET8 should be indicated.

Done

Figure 2:
Fig. 2B: gammaH2Ax should be included as a control to show that DDR is active in IR treated SET8-kd cells shown.

We did not include gH2AX during immunofluorescence as we did not find it necessary to do so because we able to observe enrichment of gH2AX in IR treated cells for both in siCON and siSET8 to the similar extent via western blotting (Figure 2C). Moreover, we had also previously observed no obvious defect in the extent of gH2AX in SET8 depleted cells (Supplementary Figure 1).

Fig. 2C: H4K20me and actin blots should be improved.

We agree the first lane (representing minus Auxin and minus IR) is not ideal, however, this condition does not affect our interpretation (i.e. that the reduction of 53BP1 cannot be explained solely by the reduction in bulk H4K20me1)--only lanes 3,4 and lane 7,8 are necessary for this interpretation as these are the only lanes representing IR treatment. We do not understand why the reviewer thinks that H4K20me1 blots are not good enough. We feel that H4K20me1 blot is as good as other blots.

Figure 3:
The authors claim to show that SET8's role in 53BP1 recruitment does not

require PCNA, this may be an over simplification. If analyzing synchronized cells, the SET8 dependent recruitment of 53BP1 after IR could be dependent upon PCNA (the phase where PCNA is most active) while this may not be the case in G1 phase.

We agree with the reviewer we have therefore toned down our wording regarding the role of PCNA. Instead of stating that PCNA is dispensable for SET8's recruitment to DSBs, we now say that role of PCNA in SET8's recruitment might be important in S-phase cells.

Figure 4:
The authors show circumstantial evidence of HDACs counteracting SET8 recruitment at DSBs. This part should be markedly expanded. The authors can for example show which HDACs are responsible and nature of interaction (direct or indirect). There is no mention of which concentrations of TSA or Na-Butyrate was used.

We apologize for this omission; we used NaB concentration and treatment as was done in Miller et al 2010 (PMID: 20802485) i.e. 16 hour incubation with 5mM NaB or 1.3µM TSA. We have added the concentration and the duration of treatment of all the drugs used in the figure legends and the methods section. We agree that the link with HDAC is interesting however we feel the suggested experiment is beyond the scope of the current manuscript manuscript.

Fig. 4E and G: SET8 and H4K20me1 abundance could be analyzed after TSA to strengthen these data.

Miller et al 2010 paper showed that treatment of cells with TSA leads to much greater increase in gH2AX than does treatment of cells with NaB (Figure 1a of the Miller et al 2010 paper); we therefore decided to restrict our ChIP experiments to NaB to avoid the complication that the change in gH2AX would have on the interpretation. We do however want to point out that we have observed consistent affect of TSA and NaB on SET8 and 53BP1 recruitment at DSBs (Figure 4A) so the suggested ChIP experiment will be redundant.

2nd Editorial Decision 05 September 2014

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

