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PARP is activated at stalled forks to mediate Mre11-dependent replication restart and recombination

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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 17 April 2009

Thank you for submitting your manuscript for consideration by the EMBO Journal. First of all, please let me apologise again for the length of time it has taken to give you a decision on your manuscript. As I told you, this was firstly due to the illness of one of our referees, who was consequently unable to return his report, and secondly because we then needed to consult external advisors - given the conflicting referees' reports. Having now received input from two editorial advisors, I am pleased to say that we are now in a position to invite you to submit a revised version of your manuscript.

As you will see from the reviewers' comments enclosed, referee 1 finds your manuscript interesting and is supportive of publication, contingent upon a number of issues. Referee 2, on the other hand, is less enthusiastic - primarily on the grounds of concerns as to the novelty of your study, since PARP has previously been implicated in replication fork restart. Given these concerns, we sought additional advice on the advance made in your study. Both external advisors felt that your manuscript provides significant novel mechanistic insight into the function of PARP at replication forks and would be suitable for publication in the EMBO Journal, should you be able to adequately address the comments of referee 1.

In the light of these recommendations, we will therefore be able to consider a revised version of your manuscript, provided you are able to answer the concerns of referee 1. I should add that it is EMBO Journal policy to allow only a single round of major revision. Therefore, acceptance of your paper will depend on the content of the next, final version.

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

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

Although PARP1 and the more recently identified has been the subject of many published papers, the roles of these proteins in mammalian DNA metabolism remains poorly defined. As noted by the authors, there is substantial circumstantial evidence linking PARP with DNA replication but not much mechanistic information. In this study, the authors provide compelling evidence that PARP1 and PARP2 are recruited to stalled replication forks and contribute to the reactivation of these forks, at least in part, by recruiting the Mre11 complex that in turn initiates recombinational repair prior to restart.

Theree are some issues that should be addressed;

PARP1 has been shown to bind to several DNA structures, including gaps.Is there a difference in binding preference for a linear gapped DNA versus the gapped fork structure (Fig. 2)? Does gap size influence binding and does RPA binding to the gap impact PARP binding?

Is the material at the top of the gel shown in Fig. 2D in the well or has it entered the gel?

The data shown in Fig. 2E should be quantitated.

What is the extent of co-localization of RPA and PAR foci (Fig. 3)?

In Fig. 6A, it appears that more Mre11 is co-iped with PARP1 in the presence of the PARP inhibitor rather than less as stated in the text. In fact there appears to be less Mre11 co-iped when cells are treated with both the PARP inhibitor and HU. This ection needs to be rewritten.

What is the extent of co-localization of Mre11 and PAR foci (Fig. 6B)?

Referee #2 (Remarks to the Author):

This study investigates the role of the PARP1 enzyme in facilitating survival of stalled replication forks and replication stress in human cells. The evidence presented shows that poly(ADP)ribose polymers are generated by PARP following hydroxyurea treatment, and that survival of human cells following HU treatment is severely affected by PARP inhibitors. PARP1 associates with sites of RPA accumulation after HU treatment, and the restart of replication forks after release from HU is at least partially dependent on PARP1 activity, as shown by DNA fibre analysis and other methods. Similar to results shown previously for DNA double-strand breaks, PARP1 is required for Mre11 localization at DNA damage sites during replication fork stalling caused by HU treatment, and the formation of RPA foci and Rad51 foci are also reduced in PARP1-deficient cells or in the presence of PARP1 inhibitors. These results lead to a general model where PARP1 and related enzymes bind to sites of stalled replication forks and recruit the MRN complex which facilitates processing and repair of the damage. The data presented are quite convincing of the conclusion that PARP1 is activated by HU treatment, although these observations are not very novel considering that PARP1 has already been shown to be an extremely rapid responder to DNA DSBs and to be required for MRN recruitment to DSB sites (JBC, 2008), and was also shown to be required for reactivation of stalled forks after HU treatment (Oncogene, 2004). In addition, one still wonders what exactly PARP1 is recognizing at the damage site - are there DSBs present at the sites of foci? What exactly does PARP1 recognize in the DNA?
Specific points:
1. If antibodies specific for gamma-H2AX are used in the experiments shown in Fig. 1 and 3 for

instance, are there DSBs observed by this method?
2. In Fig. 2d, is the fork structure actually necessary for binding by PARP1? Although the gel shift experiment does clearly show that the protein has a preference for the gapped over the ligated structure, would this also be the case for a gapped double-hairpin without a fork? Are the authors certain that the labeled substrate used in homogeneous in structure and that there are not dimer molecules present in the preparation that include regions of single-stranded DNA or DSB ends?
3. In Fig. 2e, is there a more quantitative way to do this assay, for instance using labeled precursor to specifically label the polymers?

1st Revision - authors' response 16 June 2009

Rebuttal EMBOJ-2009-70760 - point-by-point response to referees' reports:

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In the light of these recommendations, we will therefore be able to consider a revised version of your manuscript, provided you are able to answer the concerns of referee 1. I should add that it is EMBO Journal policy to allow only a single round of major revision. Therefore, acceptance of your paper will depend on the content of the next, final version.

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Theree are some issues that should be addressed:

PARP1 has been shown to bind to several DNA structures, including gaps.Is there a difference in binding preference for a linear gapped DNA versus the gapped fork structure (Fig. 2)? Does gap size influence binding and does RPA binding to the gap impact PARP binding?

We agree with the referee that it is interesting to understand what exactly PARP1 recognises at a stalled fork (this was also brought up by referee 2). To address this issue we made stalled constructs containing different gap lengths and measured the kinetics of PARP1 activation as the referee suggested. The new results show that PARP1 is efficiently activated by a gap length up to 4 nucleotides and that the activation drops drastically with a 8 nucleotide gap.

We find that the stalled construct activates PARP1 to the same extent as the sonicated DNA positive control, which will include SSBs on linear DNA. Thus, a forked structure is an excellent substrate for PARP1.

The referee asks if RPA binding to the gap impact PARP binding. RPA is a trimer and altogether has 4 ssDNA binding domains. A single RPA trimer is likely to bind single-stranded nucleotides greater than 24 nt long (Cai et al Biochemistry. 2007;46(28):8226-33). We find that increasing the gap size from 4 to 8 nucleotides decreases PARP activity. Thus, RPA is not likely to be able to bind

the 4 nucleotide gap that activates PARP1 in our assays. Principally, it would be interesting to see if the presence of RPA would allow PARP1 be activated by longer gaps. Unfortunately, the three subunits of RPA are not commercially available and purifying the three subunits to make such experiments is outside the remit of the current report (it is already extensive).

We would like to thank the referee making us do these experiments as it improves the paper extensively. We can now say that only a subset of stalled forks that do not include extensive ssDNA gaps are activated by PARP1. This result fits well with our finding that only a subset of Mre11 foci co-localise with PAR foci (new Figure 6C) and that not all Mre11 foci depend on PARP activity (Figure 6D). This also likely explains why we still see some ssDNA formation (scored as RPA foci) after PARP inhibition.

Is the material at the top of the gel shown in Fig. 2D in the well or has it entered the gel?

The band at the very top of the gel (seen most clearly in lane 6) is in the well. The reason for this is that the PAR polymers (formed by the activated PARP) are extremely long and branched and do not migrate into the gel. However the bands we refer to in the text have clearly run into the gel (seen most clearly in lanes 2, 3 and 7. Increasing the concentration of PARP present in the reaction shifts the PARP-DNA bands upward in the gel, presumably as a result of multimers of PARP protein binding. At the highest concentrations this results in the PARP-DNA complex being stuck in the wells

The data shown in Fig. 2E should be quantitated.

We have quantified this data and mention in the text that there is a 5-fold increase in PAR formation. However, the data in 2E say very little about the kinetics of PARP1 activation. We therefore performed a kinetic analysis of PARP1 activity in presence of the various stalled fork structures and control DNA. The activity was thus quantified and is presented in the revised version.

What is the extent of co-localization of RPA and PAR foci (Fig. 3)?

We have now quantified the extent of co-localisation of PAR and RPA foci in PARP1+/+ MEFs and included the results as a new Figure 3B. As indicated in the pictures the co-localisation is almost perfect.

In Fig. 6A, it appears that more Mre11 is co-iped with PARP1 in the presence of the PARP inhibitor rather than less as stated in the text. In fact there appears to be less Mre11 co-iped when cells are treated with both the PARP inhibitor and HU. This ection needs to be rewritten.

We spotted this mistake ourselves and sent a new Figure 6A to the editor at a late stage. The Figure 6A was mislabelled (the HU and PARP inhibitor) had swapped place. The revised figure 6 does not have this mistake.

What is the extent of co-localization of Mre11 and PAR foci (Fig. 6B)?

We have done more experiments to address this question in more detail. In contrast to RPA, the co-localisation isn't perfect and only 70% of PAR foci co-localise with Mre11 foci. The imperfect co-localisation is also in agreement with the data showing that PARP inhibitors do not fully prevent HU-induced Mre11 foci and that ssDNA formation (scored by RPA foci) is not fully inhibited in PARP1-/- MEFs. This could mean that PARP1 is only recognising a portion of the stalled forks (the portion not including extensive ssDNA regions as realised from the experiment above suggested by the referee).

We are grateful to this referee as the two suggested experiments have made a substantial improvement to the manuscript, we now have a greater understanding that (1) PARP1 is involved in recognising a subset of stalled forks and (2) the forks recognised by PARP1 are those that do not contain extensive ssDNA gaps.

Referee #2 (Remarks to the Author):

This study investigates the role of the PARP1 enzyme in facilitating survival of stalled replication forks and replication stress in human cells. The evidence presented shows that poly(ADP)ribose polymers are generated by PARP following hydroxyurea treatment, and that survival of human cells following HU treatment is severely affected by PARP inhibitors. PARP1 associates with sites of RPA accumulation after HU treatment, and the restart of replication forks after release from HU is at least partially dependent on PARP1 activity, as shown by DNA fibre analysis and other methods. Similar to results shown previously for DNA double-strand breaks, PARP1 is required for Mre11 localization at DNA damage sites during replication fork stalling caused by HU treatment, and the formation of RPA foci and Rad51 foci are also reduced in PARP1-deficient cells or in the presence of PARP1 inhibitors. These results lead to a general model where PARP1 and related enzymes bind to sites of stalled replication forks and recruit the MRN complex which facilitates processing and repair of the damage. The data presented are quite convincing of the conclusion that PARP1 is activated by HU treatment, although these observations are not very novel considering that PARP1 has already been shown to be an extremely rapid responder to DNA DSBs and to be required for MRN recruitment to DSB sites (JBC, 2008), and was also shown to be required for reactivation of stalled forks after HU treatment (Oncogene, 2004). In addition, one still wonders what exactly PARP1 is recognizing at the damage site - are there DSBs present at the sites of foci? What exactly does PARP1 recognize in the DNA?

The referee suggests the novelty of this study isn't high. Indeed, it has very recently been shown that inhibition of PARP influence the recruitment of Mre11 to laser-induced damage. Laser induced damage these authors use do not only cause DSBs, but completely obliterate ëfry' the nucleus into irreparable lesions that completely disrupt the chromatin structure. The oncogene paper showed that PARP inhibited cells do not enter into the cell cycle by FACS after HU treatments. They speculate that this involves replication restart, but provided absolutely no proof of it ñ it could equally well be that this portion of cells died or that PARP inhibits new origin firing. So by no means did they show that PARP is involved in replication restart. We agree that there are these two experiments in the literature supporting our model. Here, we provide additional experiments (38 panels in 10 Figures + 17 panels in 6 supplement figures) that actually prove what can be speculated from these two earlier results.

The referee wants to know what exactly PARP1 is recognising. We have addressed this in our additional experiments, suggesting it is a gap smaller than 4 NTs. As no one has been able to see what a stalled replication fork exactly looks like even in lower eukaryotes where you can isolate replication intermediates. It is therefore impossible in a report of this nature to make further comment on what PARP is recognising.

Specific points:
1. If antibodies specific for gamma-H2AX are used in the experiments shown in Fig. 1 and 3 for instance, are there DSBs observed by this method?

Firstly it is a debateable point as to whether gamma H2AX antibodies really do recognise only DSBs or rather they show areas of disrupted chromatin structure, therefore this would not be the experiment to use to look for DSBs.

In addition we see that PARP is activated by HU stalling already after 3 hours and that it is required to restart the forks at this time. We and other have previously shown that no DSBs are formed at this point.

As this is an important point we addressed this specifically using a drug that does not induce DSBs at all, which is thymidine. Thymidine slows replication forks without forming DSBs even after prolonged treatments as we published in numerous previous papers. However, PARP is triggered by thymidine even quicker and more pronounced that after HU treatments, which prove that there is no need for DSBs to form. However, it is possible that PARP1 may attract Mre11 also to collapsed forks and we include this as a plausible model in discussions and in our final model (Figure 10).

2. In Fig. 2d, is the fork structure actually necessary for binding by PARP1? Although the gel shift experiment does clearly show that the protein has a preference for the gapped over the ligated structure, would this also be the case for a gapped double-hairpin without a fork? Are the authors

certain that the labeled substrate used in homogeneous in structure and that there are not dimer molecules present in the preparation that include regions of single-stranded DNA or DSB ends?

It is well established that PARP1 can bind non-forked structures. We have included a much more detailed analysis of the gapped regions influence of PARP1 activity in the revised version as discussed above.

3. In Fig. 2e, is there a more quantitative way to do this assay, for instance using labeled precursor to specifically label the polymers?

Yes, there is and we've done a proper kinetics assay in the revised version.

