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Rad51 replication fork recruitment is required for DNA damage tolerance

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 13 December 2012

Thank you for submitting your manuscript on replication fork roles of yeast Rad51 for consideration by The EMBO Journal. Three expert referees have now evaluated it, and as you will see from their reports copied below, they all consider your results potentially interesting and important but raise a number of substantive issues that would need to be clarified before publication may be warranted. Since these points appear in principle all addressable, I shall be happy to give you an opportunity to respond to these concerns in the form of a revised version of this manuscript. I should nevertheless stress that some points, such as referee 1's key concern regarding functionality of tagged Rad51 and Rad52 proteins, have the potential to undermine the current conclusions, and eventual acceptance of the study will therefore depend on convincing the referees with additional data on these critical issues. In this respect, please also keep in mind that it is our policy to allow only a single round of major revision and that it is therefore important to carefully and comprehensively respond to all points at this stage. When preparing your letter of response to the referees' comments, please also remember that this will form part of the Review Process File, and will therefore be available online to the community.

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.

Thank you for the opportunity to consider this work, and please do not hesitate to contact me in case you should have any additional question regarding this decision or the reports. I look forward to

your revision.

———

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

This manuscript follows observations in Xenopus (Lopes, Costanzo) and mammalian cells (Jasin, Helleday) regarding a replication fork escort function of Rad51 protein. The authors report the first evidence in yeast by adapting the ChEC method of Laemmli to study recombination proteins. They make a number of important conclusions that significantly extend our knowledge of the replication fork escort function of Rad51. The ChEC method provides independent evidence for Rad51 and Rad52 recruitment. The recruitment of Rad52 and Rad51 is coupled to replication and this early recruitment is required for later repair. The S-phase DDR restricts recombination in S-phase, and CDK is required for repair in G2.

There are a number of concerns that the authors would need to address, in order to ascertain that these conclusions are supported by the data.

1) A significant problem is the changing concentrations of MMS from experiment to experiment. This leaves the impression that very selected data are shown. For example, the MN fusions of Rad51 and Rad52 are shown to be proficient to grow in the presence of 0.006% and 0.014% MMS (Fig. 1B), but the experiments are conducted at other concentrations (Fig. 1C 0.05%). Are the fusions functional at the experimental conditions, specifically 0.05% MMS? This needs to be clarified.

The authors state on page 6 that the fusions "were as proficient as wild type", but Figure S1A shows HU sensitivity for the Rad52 fusion at 150 mM. This needs to be acknowledged.

I have strong concerns that phenotypes of the fusions proteins interfere with the analysis and interpretation. (See also #3)

2) Figure 3B: A major result appears to be the higher baseline of Rad52-YFP foci in the sgs1 exo1 double mutant. Please comment.

The authors should also consider that gaps may potentially be or need to be enlarged for Rad51 nucleation.

3) There is no information, whether the Rad51 and Rad52 YFP fusions are functional. These proteins are notoriously difficult to tag without affecting their function (See point #1).

I have strong concerns that phenotypes of the fusions proteins interfere with the analysis and interpretation.

4) Fig. 4C: Is there any effect of the Ca++ alone?

5) I find it puzzling that there are no Rad52-YFP foci in S-phase, but a strong ChEC signal; whereas, there are small RAD51-YFP foci, but a weaker ChEC signal than for Rad52. This relates to the concerns about the fusion. Is this a real observation or an artifact of the fusions used?

6) The interpretation of Fig. 6C and Fig. S4 is questionable. The authors state that lack of Mec1 or Rad53 "partially releases" HR inhibition, but the effect is stronger than in the mrc1 AQ rad9 double mutant.

Also, the big difference in the rad53 chk1 and mec1 tel1 strains seems to be the very high background of Rad52 foci, which needs to be discussed. The mec1 tel1 results appear to be uninterpretable in the context of the time course. There is not much increase from 30 min to 180 min, with the exception of 180 min with 0.01% MMS.

7) Discussion:

Jachymczyk, WJ (1977 Mutat Res, 1979 MGG) showed that high concentrations of MMS (0.5%) induce DSB, but not lower concentrations.

Page 15, line 15: The authors claim to show the first evidence of Rad51 and Rad52 binding to unperturbed replication forks, and proceed to discuss the previous literature that demonstrated this point. This claim should be removed.

The replication-dependence may suggest an involvement of sister chromatid cohesion. Such a connection could further strengthen this point.

Additional points:
8) Abstract: The sentence starting with "Notably" is unclear and should be rephrased. I understood the sentence only after reading the entire manuscript, but that should not be necessary to understand the abstract.

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16) Page 12, line 14: The ratio of completing DNA replication needs to be explained better.

17) Page 13, line 9: "Inhibition of recombination": What do you mean, inhibition of focus formation? If so, please be precise.

Referee #2 (Remarks to the Author):

In this manuscript the authors have analyzed the contributions of homologous recombination (HR) factors to the processing of replication problems in yeast. They report evidence for functions of the HR proteins Rad51 and Rad52 in undisturbed replication as well as after treatment with the alkylating agent MMS. These two functions appear to be linked by a loading of the proteins onto DNA at replication forks that is independent of DNA damage, but becomes a requirement for the processing of damage by HR later on. Intriguingly, they find that this type of MMS-induced HR is limited to the G2 phase of the cell cycle, i.e. long after the HR factors have been loaded onto the DNA. The authors discuss their findings in the context of a model where the loading of Rad51 and Rad52 could be connected to the restart of DNA replication downstream of lesions, while a combination of the replication checkpoint and CDK activity would delay the actual processing until G2/M.

The results presented here are noteworthy and interesting, although the manuscript is difficult to read. This difficulty in following the authors' arguments is probably more due to the complicated subject matter than a lack of quality of the writing itself. The most significant point in my opinion is the description of a timed reaction that delays HR (despite the presence of the relevant factors) until G2/M. Demonstrating the presence of HR factors even at undisturbed forks would have significant implications for models of how HR is activated, although unfortunately the mechanistic details of how a separation between HR factor loading and activation would be accomplished remain unexplored.

Overall, I was largely convinced by the data, although the differences observed between relevant states are sometimes minor, which makes me wonder a bit about the magnitude of the effects described here. There are a few specific concerns that should be addressed:

1. I would recommend re-writing the introduction to focus more about HR rather than damage tolerance. The intro is set up in such a way that one expects the authors to address the question of how the RAD6 pathway cooperates with HR in damage bypass. This question is not at all picked up in the results, where it is all about HR, but we do not learn whether or not the observed phenomena are linked in any way to RAD6-dependent template switching. This lack of a connection could be perceived as a hole in the authors' arguments unless they re-focus their introduction.

2. The authors conclude from their data that Rad52-dependent Rad51 loading onto chromatin is coupled to replication forks. I do not quite follow this argument. It appears to me that they have indeed shown that this reaction must occur in S phase and cannot be delayed to G2/M, but unless I have missed something, I am not aware of any evidence that this might not happen post-replicatively (while cells are still in S phase). This could be due to a time window or the cell cycle stage rather than the coupling to a specific DNA structure. This point is important for the authors' model that links the loading of HR factors to re-priming.

3. Can the authors exclude that the MNase assay measures a de novo association of HR factors with ssDNA during the Ca++ treatment rather than the pre-association that dates back to the passing of the replication fork? In MMS-treated cells, the analysis of Rad51 or Rad52 foci provides a good level of confidence about the presistence of the HR factors at the fork, but as there are no foci in undamaged cells, how can the authors be sure that they do not measure a new loading of the proteins? Addressing this point is essential for supporting their model of how the loading versus activity of HR factors is timed.

4. Along similar lines, why wouldn't there be Rad51/Rad52 foci in undisturbed S phase cells regularly, if the association of these HR factors is independent of damage and doesn't increase after MMS treatment? To my knowledge, these S-phase-associated foci largely appear after damage treatment and only rarely in undamaged cells, where their appearance has been explained by spontaneous fork problems.

5. In Figure 4D the percentage of X-shaped structures appears abnormally high compared to similar assays in other strains. It would be preferable to have a direct comparison of two experiments performed side-by-side in order to assess whether this variability is relevant or not.

6. On page 6 (lower third), it would be preferable to write "(down to 1 kb)" rather than "(up to 1 kb)", as the size of the fragments is getting progressively smaller, not larger.

Referee #3 (Remarks to the Author):

Resume
In this manuscript, the authors have used a combination of various techniques to investigate the role of homologous recombination during the replication of a damaged template. The authors have developed an elegant approach, based on Chromatin Endogenous Cleavage (ChEC), to demonstrate that the recombination factors Rad52 and Rad51 associate to replication forks during unchallenged S-phase. During replication of alkylated-DNA templates, Rad52 and Rad51 associate to non-Double-strand-break DNA lesions during S-phase and G2, but form intense foci only when DNA replication is completed. Binding of Rad52 to replication forks is required to promote S-phase progression in presence of MMS, but also to promote Rad51 foci in G2 phase. Based on these data, the authors have proposed a model in which the completion of DNA replication upon DNA-damage is achieved by homologous recombination in a two-steps process. First, binding of recombination factors to replication forks allows the bypass of the DNA lesion, second homologous recombination promotes the repair of the single stranded DNA gap left behind the moving fork. Finally, the authors have provided further data to establish that the second step of damage tolerance by recombination is promoted by CDK1 and restricted to the G2/M phase by DNA replication checkpoint pathways. In

the discussion, the authors have speculated on two distinct functions for homologous recombination: a replicative and non-repair function at replication forks, and a repair function in G2/M.

In general, the data presented are of good quality and not over-interpreted (except for the checkpoint part, see major point 3). The model proposed is appealing and provides novel insights in the mechanism of damage tolerance by homologous recombination. The model also reinforces previous observations made on the structure of damaged replication forks, visualized by electronic microscopy in the absence of recombination (published by Hashimoto et al. 2010). To my knowledge, the experiments provided in this manuscript are the most convincing to support a role of homologous recombination in the bypass of DNA lesions at the replication fork, and not only behind the moving fork. However, I might suggest the authors to perform a couple of additional experiments to reinforce their claim, especially to clarify their words on distinct functions of homologous recombination (non-repair function during replication, see major point 2).

Major points

1. The most striking data is the fact that Rad52 has to bind DNA lesions in S-phase to allow the recruitment of Rad51 to DNA lesions (Fig 2G) and the formation of Rad51 foci (Fig 3C) in G2/M. These data are central to conclude that binding of recombination proteins to the replication fork is a prerequisite for the repair of ssDNA gaps in G2. Nonetheless, the absence of Rad51 foci or DNA binding in G2/M could be also explained by a reduced expression level of Rad51, upon MMS treatment. This point should be easy to address. Moreover, the model proposed implies that the strain expressing Rad52 only in G2 (G2::cRAD52, defective for Rad51 binding to DNA and Rad51 foci formation in G2/M) should be also defective for the formation of sister-chromatid junctions, as visualized by 2D-gels on Figure 7B) and should accumulate unrepaired gaps. Such experiments would reinforce their conclusion.

2. Throughout the text, the authors refer to Rad52 foci as a repair function and to Rad52 binding to replication forks as a non-repair function. It is unclear what their cases to support such conclusion are. For example, page 15, lines 6 "Rad52 and Rad51 bind to unperturbed forks and promote replication through alkylated DNA by repair independent mechanisms". Do the authors refer to the repair of ssDNA gaps containing the DNA lesion in G2 and bypass of DNA lesions at the replication fork? If so, they should clarify their words.

3. Based on the analysis of Rad52 foci during S-phase in response to MMS treatment in checkpoint mutants, the authors conclude that the Mrc1-branch and Rad9-branch of replication checkpoints prevent recombinational repair during S-phase (Fig 6 and S4). Thus, the authors claim that the repair of ssDNA gaps by recombination is restricted to G2/M by the replication checkpoint. I found the data presented poorly convincing. First, some of the mutants studied present high level of spontaneous Rad52 foci, that renders kinetic studies difficult to interpret (Fig S4, sml1 rad53 chk1 and sml1 mec1 tel1). Second, the accumulation of Rad52 foci during S-phase in the mrc1AQ rad9 double mutant, could be the consequence of the dynamic of DNA replication being affected by the loss of checkpoint function (more origins activated, more recombinogenic structures formed at replication forks..). Thus, I found difficult, based on the data provided, to conclude that the replicative checkpoint restricts the repair of ssDNA gaps to the G2/M phase. I would suggest the authors to provide stronger evidences to support their claim.

Minor points

Page 10, lines 8: I don't think that "reverted" is the right word here. I would suggest "converted".
Page 10, lines 22: "note that in the presence of MMS....of the Y-arc". What the authors meant here is unclear.
Page 12, line 15: the reference to Meister et al. 2005 for the bud-to mother size ratio is meaningless.
Page 15, lines 25 'However, natural impediments are unlikely.....fork progression". What is the rational to claim this ?
Page 19, lines 12: refer to Figure S6 instead of S7.
Figure 5, panel C: the histogram on the top left is too small

1st Revision - authors' response 18 February 2013

Referee #1 *(Remarks to the Author):*

"Rad51 replication fork recruitment is required for DNA damage tolerance" [manuscript # EMBOJ-2012-83909]

This manuscript follows observations in Xenopus (Lopes, Costanzo) and mammalian cells (Jasin, Helleday) regarding a replication fork escort function of Rad51 protein. The authors report the first evidence in yeast by adapting the ChEC method of Laemmli to study recombination proteins. They make a number of important conclusions that significantly extend our knowledge of the replication fork escort function of Rad51. The ChEC method provides independent evidence for Rad51 and Rad52 recruitment. The recruitment of Rad52 and Rad51 is coupled to replication and this early recruitment is required for later repair. The S-phase DDR restricts recombination in S-phase, and CDK is required for repair in G2.

There are a number of concerns that the authors would need to address, in order to ascertain that these conclusions are supported by the data.

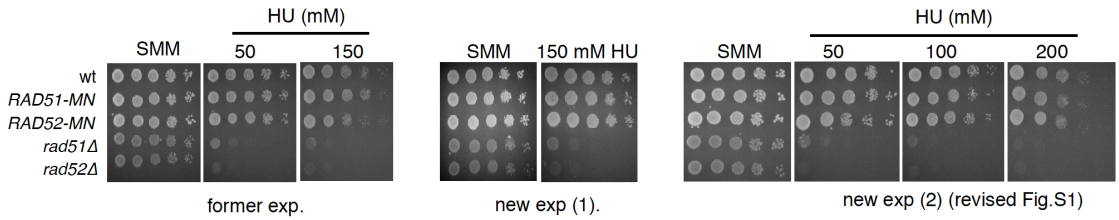
1) A significant problem is the changing concentrations of MMS from experiment to experiment. This leaves the impression that very selected data are shown. For example, the MN fusions of Rad51 and Rad52 are shown to be proficient to grow in the presence of 0.006% and 0.014% MMS (Fig. 1B), but the experiments are conducted at other concentrations (Fig. 1C 0.05%). Are the fusions functional at the experimental conditions, specifically 0.05% MMS? This needs to be clarified.

The functionality of the chimeras was tested by serial dilutions on media containing MMS. At higher MMS concentrations (0.033% or 0.05%) this chronic damage kills all cells, even the wild-type cells (data not shown).

The functionality of the chimeras at the experimental conditions has been analyzed by viability assays. Both Rad52-MN and Rad51-MN were as functional as the wild type. See new Figure 1C.

The authors state on page 6 that the fusions "were as proficient as wild type", but Figure S1A shows HU sensitivity for the Rad52 fusion at 150 mM. This needs to be acknowledged.

The subtle difference between the chimera and the wild type is due to pipetting errors. See bellow two additional independent experiments as well as the former one. In this version we have replaced the former one with exp. 2. See new Figure S1A.



I have strong concerns that phenotypes of the fusions proteins interfere with the analysis and interpretation. (See also #3)

Under our experimental conditions Rad52-MN and Rad51-MN displayed the same resistance to DNA damaging agents as the wild-type proteins. This has been tested with different types of DNA damaging agents using chronic (MMS, Zeo, HU, CPT) and acute (UV light) doses (Figures 1B, 1C and S1A), and for MMS and Zeo, upon exposure to both chronic and acute treatments (Figures 1B, 1C and S2). We have also shown that the chimera cRad52 is as functional as Rad52 under our experimental conditions even in the presence of Rad51-MN (Figures 2C and S2). Therefore, and as far as we have checked, our results cannot be explained by specific phenotypes of the fusions

proteins.

2) Figure 3B: A major result appears to be the higher baseline of Rad52-YFP foci in the sgs1 exo1 double mutant. Please comment.
The authors should also consider that gaps may potentially be or need to be enlarged for Rad51 nucleation.

Done as requested; see new second paragraph at the Results section "*The recruitment of Rad51 to replicating chromatin is required for MMS-induced HR repair".* Actually, the idea about gaps enlargement was already discussed as a putative mechanism by which recombinational repair is prevented during S phase.

3) There is no information, whether the Rad51 and Rad52 YFP fusions are functional. These proteins are notoriously difficult to tag without affecting their function (See point #1).

Note that, to overcome this problem, the HR foci analyses were performed with strains expressing both the chimera and the wild-type protein. We have now included the functionality of these strains in response to both MMS and zeocine under our experimental conditions (See new Figure S3A, and text in the new first paragraph at the Results section "*The recruitment of Rad51 to replicating chromatin is required for MMS-induced HR repair".* These strains are fully functional in DNA repair. Please also note that the functionality of YFP-Rad51/Rad51 was not affected by the presence of cRad52 (Figure S3D).

I have strong concerns that phenotypes of the fusions proteins interfere with the analysis and interpretation.

Tagging recombination proteins with YFP or MN is a useful approach to follow the binding of these proteins to replicative DNA lesions. We agree that this approach can be limited when the functionality of the repair protein is altered. However, as mentioned before, and as far as we have checked, our strains are fully proficient on DNA repair. Thus, our results are unlikely affected by the use of fusion proteins.

4) Fig. 4C: Is there any effect of the Ca++ alone?

No; see new Figure S4A.

5) I find it puzzling that there are no Rad52-YFP foci in S-phase, but a strong ChEC signal; whereas, there are small RAD51-YFP foci, but a weaker ChEC signal than for Rad52. This relates to the concerns about the fusion. Is this a real observation or an artifact of the fusions used?

The fact that the faint, small foci are detected with YFP-Rad51, but not with Rad52-YFP, might reflect a higher number of Rad51 molecules at the ssDNA gaps. We have not detected such a correlation with the proteins fused to the MN with the ChEC assays. As we have shown, Rad51-MN and Rad52-MN are fully functional in DNA repair. However, the ability of the fusion proteins to cleave the DNA in our assay is very likely to be strongly influenced by the nuclease activity of the MN domain at the DNA in the context of the fusion protein (DNA accessibility of the MN domain, MN folding…). As indicated in the text (Results Section "*Rad52 and Rad51 bind to replication forks regardless of the presence of DNA damage"*), the weaker ChEC signal obtained with Rad51-MN as compared to Rad52-MN (and observed both at the bulk DNA and at the fork) might reflect a lower nuclease activity of the former. YFP and MN are different functional domains whose activities are unlikely to be influenced by Rad51 and Rad52 in the same way.

6) The interpretation of Fig. 6C and Fig. S4 is questionable. The authors state that lack of Mec1 or Rad53 "partially releases" HR inhibition, but the effect is stronger than in the mrc1 AQ rad9 double mutant.

Also, the big difference in the rad53 chk1 and mec1 tel1 strains seems to be the very high background of Rad52 foci, which needs to be discussed. The mec1 tel1 results appear to be uninterpretable in the context of the time course. There is not much increase from 30 min to 180 min, with the exception of 180 min with 0.01% MMS.

Corrected as suggested. See the revised Results section "*The replicative checkpoint prevents the assembly of MMS-induced HR repair centers during DNA replication"* and the revised Discussion section *"MMS-induced recombinational repair is cell cycle regulated by the replicative checkpoint and the CDK activity of Cdc28"*.

7) Discussion:
Jachymczyk, WJ (1977 Mutat Res, 1979 MGG) showed that high concentrations of MMS (0.5%) induce DSB, but not lower concentrations.

Included as suggested; see new Discussion section "*The alkylating agent MMS generates replicative, non-DSBs recombinogenic DNA lesions"*.

Page 15, line 15: The authors claim to show the first evidence of Rad51 and Rad52 binding to unperturbed replication forks, and proceed to discuss the previous literature that demonstrated this point. This claim should be removed.

Removed as suggested.

The replication-dependence may suggest an involvement of sister chromatid cohesion. Such a connection could further strengthen this point.

Cohesin in yeast has been recently shown to be loaded at active replication forks, spread along DNA as forks progress, and be required for MMS-induced DDT (e.g., X formation) (Tittel-Elmer et al. 2012; Mol Cell 48:1-11), establishing a strong connection between DDT and sister chromatid cohesion. Even though it would be interesting to go further in the mechanisms by which cohesin promotes DDT (role of cohesin on Rad52 loading/stabilization, location of cohesin at the fork and/or at gaps left behind the fork, requirement for HR foci formation…), it is a huge study that is beyond the scope of this work.

Additional points:
8) Abstract: The sentence starting with "Notably" is unclear and should be rephrased. I understood the sentence only after reading the entire manuscript, but that should not be necessary to understand the abstract.

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12) Page 8, line 1: Remove 'strongly'.

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13) Page 8, line 8: Why does the sentence start with "Additionally"?

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14) Page 10, 3rd to last line: The description is unclear. Do you mean resulting in Y-shaped molecules at the inflection point?

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Explained as suggested.

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The results presented here are noteworthy and interesting, although the manuscript is difficult to read. This difficulty in following the authors' arguments is probably more due to the complicated subject matter than a lack of quality of the writing itself. The most significant point in my opinion is the description of a timed reaction that delays HR (despite the presence of the relevant factors) until G2/M. Demonstrating the presence of HR factors even at undisturbed forks would have significant implications for models of how HR is activated, although unfortunately the mechanistic details of how a separation between HR factor loading and activation would be accomplished remain unexplored.

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1. I would recommend re-writing the introduction to focus more about HR rather than damage tolerance. The intro is set up in such a way that one expects the authors to address the question of how the RAD6 pathway cooperates with HR in damage bypass. This question is not at all picked up in the results, where it is all about HR, but we do not learn whether or not the observed phenomena are linked in any way to RAD6-dependent template switching. This lack of a connection could be perceived as a hole in the authors' arguments unless they re-focus their introduction.

Revised as suggested.

2. The authors conclude from their data that Rad52-dependent Rad51 loading onto chromatin is coupled to replication forks. I do not quite follow this argument. It appears to me that they have indeed shown that this reaction must occur in S phase and cannot be delayed to G2/M, but unless I have missed something, I am not aware of any evidence that this might not happen post-replicatively (while cells are still in S phase). This could be due to a time window or the cell cycle stage rather than the coupling to a specific DNA structure. This point is important for the authors' model that

links the loading of HR factors to re-priming.

It is true that our results demonstrate that Rad52 and Rad51 have to be loaded during DNA replication and not necessarily at the fork. However, the fact that they interact with the fork both in the absence and presence of MMS and are required for replication fork advance through alkylated DNA makes us favor the idea that their loading onto chromatin is coupled to replication forks. We have clarified this point in this revised version.

3. Can the authors exclude that the MNase assay measures a de novo association of HR factors with ssDNA during the Ca++ treatment rather than the pre-association that dates back to the passing of the replication fork? In MMS-treated cells, the analysis of Rad51 or Rad52 foci provides a good level of confidence about the presistence of the HR factors at the fork, but as there are no foci in undamaged cells, how can the authors be sure that they do not measure a new loading of the proteins? Addressing this point is essential for supporting their model of how the loading versus activity of HR factors is timed.

As shown in new Figure S4A, Ca++ by itself did not alter the pattern of replication intermediates. We have also shown that the ChEC signal generated by the chimeras was specific as it was not observed with MN alone (Figure S1B) and in the case of Rad51-MN was dependent on the presence of Rad52 (Figures 1E and 4F). In addition, and as shown in Figure 2G, the absence of Rad52 in S phase prevented the binding and cleavage of Rad51-MN to chromatin in *G2::cRAD52* cells despite in G2/M these cells have Rad52, Rad51-MN, ssDNA, and are treated with Ca++. This result argues against the possibility that the ChEC signal measured the binding of HR factors to ssDNA during the Ca++ treatment. Our results in yeast with the Rad51-MN and Rad52-MN chimeras are consistent with analyses in Xenopus and mammalian cells showing that the presence of genotoxic agents hardly increases the recruitment of Rad51. These arguments are included in the revised version.

4. Along similar lines, why wouldn't there be Rad51/Rad52 foci in undisturbed S phase cells regularly, if the association of these HR factors is independent of damage and doesn't increase after MMS treatment? To my knowledge, these S-phase-associated foci largely appear after damage treatment and only rarely in undamaged cells, where their appearance has been explained by spontaneous fork problems.

Note that we report two types of cells with YFP-Rad51 foci in response to MMS. 1) Cells with 1-2 bright foci, which accumulated in G2/M and thereby earlier at low dose; these foci are similar to those observed with Rad52-YFP and are also induced with Zeocine. Therefore, they are likely repair centers. 2) Cells with small, speckled foci that accumulated in S phase, were dose dependent, and importantly, did not appear in response to zeocine. The accumulation of these faint foci correlated with the ChEC signal by HR proteins that occurred in S phase in response to MMS. Therefore, they likely reflect the binding of HR proteins to ssDNA left behind the fork. The lack of HR foci in unperturbed cells suggests that the amount of Rad52/Rad51 at forks might be insufficient and/or too diffuse to generate a detectable signal. We have explained this better in Results section "*MMS-induced HR repair occurs after DNA replication".*

5. In Figure 4D the percentage of X-shaped structures appears abnormally high compared to similar assays in other strains. It would be preferable to have a direct comparison of two experiments performed side-by-side in order to assess whether this variability is relevant or not.

Done as requested. As can be seen in new Figure S4B the increase in X-shaped molecules at 45 minutes wit Ca++ is not significant.

6. On page 6 (lower third), it would be preferable to write "(down to 1 kb)" rather than "(up to 1 kb)", as the size of the fragments is getting progressively smaller, not larger.

Changed as suggested.

Referee #3 (Remarks to the Author):

Resume

In this manuscript, the authors have used a combination of various techniques to investigate the role of homologous recombination during the replication of a damaged template. The authors have developed an elegant approach, based on Chromatin Endogenous Cleavage (ChEC), to demonstrate that the recombination factors Rad52 and Rad51 associate to replication forks during unchallenged S-phase. During replication of alkylated-DNA templates, Rad52 and Rad51 associate to non-Double-strand-break DNA lesions during S-phase and G2, but form intense foci only when DNA replication is completed. Binding of Rad52 to replication forks is required to promote S-phase progression in presence of MMS, but also to promote Rad51 foci in G2 phase. Based on these data, the authors have proposed a model in which the completion of DNA replication upon DNA-damage is achieved by homologous recombination in a two-steps process. First, binding of recombination factors to replication forks allows the bypass of
the DNA lesion, second homologous recombination promotes the repair of the single stranded DNA gap left behind the moving fork. Finally, the authors have provided further data to establish that the second step of damage tolerance by recombination is promoted by CDK1 and restricted to the G2/M phase by DNA replication checkpoint pathways. In the discussion, the authors have speculated on two distinct functions for homologous recombination: a replicative and non-repair function at replication forks, and a repair function in G2/M.

In general, the data presented are of good quality and not over-interpreted (except for the checkpoint part, see major point 3). The model proposed is appealing and provides novel insights in the mechanism of damage tolerance by homologous recombination. The model also reinforces previous observations made on the structure of damaged replication forks, visualized by electronic microscopy in the absence of recombination (published by Hashimoto et al. 2010). To my knowledge, the experiments provided in this manuscript are the most convincing to support a role of homologous recombination in the bypass of DNA lesions at the replication fork, and not only behind the moving fork. However, I might suggest the authors to perform a couple of additional experiments to reinforce their claim, especially to clarify their words on distinct functions of homologous recombination (non-repair function during replication, see major point 2).

Major points

1. The most striking data is the fact that Rad52 has to bind DNA lesions in S-phase to allow the recruitment of Rad51 to DNA lesions (Fig 2G) and the formation of Rad51 foci (Fig 3C) in G2/M. These data are central to conclude that binding of recombination proteins to the replication fork is a prerequisite for the repair of ssDNA gaps in G2. Nonetheless, the absence of Rad51 foci or DNA binding in G2/M could be also explained by a reduced expression level of Rad51, upon MMS treatment. This point should be easy to address.

Done as requested. As shown in new Figures 2G and S3C, the strains show similar amounts of Rad51 in the presence of MMS as determined by western blot, excluding differences in the level of Rad51 as the cause of the absence of YFP-Rad51 foci or Rad51-MN binding in *G2::cRAD52* cells as compared to the wild type.

Moreover, the model proposed implies that the strain expressing Rad52 only in G2 (G2::cRAD52, defective for Rad51 binding to DNA and Rad51 foci formation in G2/M) should be also defective for the formation of sister-chromatid junctions, as visualized by 2D-gels on Figure 7B)

Done as requested. As shown in new Figure 3D, *G2::cRAD52 sgs1*Δ cells are defective in the formation of MMS-induced X-shaped molecules.

and should accumulate unrepaired gaps. Such experiments would reinforce their conclusion.

Done as requested. As shown in new Figure 3E, MMS-induced Rfa1-YFP foci accumulate in *G2::cRAD52* and *rad52*Δ cells treated for 1 hour with 0.01% MMS and released in fresh medium to allow the repair of the ssDNA gaps. Interestingly, the analysis suggests that Rad52 during S phase is required for ssDNA gap repair but not for the recruitment of the lesion to the repair center. This observation has also been included.

2. Throughout the text, the authors refer to Rad52 foci as a repair function and to Rad52 binding to replication forks as a non-repair function. It is unclear what their cases to support such conclusion

are. For example, page 15, lines 6 "Rad52 and Rad51 bind to unperturbed forks and promote replication through alkylated DNA by repair independent mechanisms". Do the authors refer to the repair of ssDNA gaps containing the DNA lesion in G2 and bypass of DNA lesions at the replication fork? If so, they should clarify their words.

Clarified as suggested. Now it reads in the second section of Discussion: "Rad52 and Rad51 are required for replication fork progression through alkylated DNA (Figures 2D-E) (Vázquez *et al*, 2008; Alabert *et al*, 2009), a function that is usually attributed to their DNA repair activities. In contrast to this view, we show that Rad52 is bound to MMS-induced non-DSB DNA lesions when replication is largely completed (Figure 2A) and that HR foci do not form until G2 (Figure 5). These results suggest that Rad52 and Rad51 help replication forks bypass DNA lesions by repair independent mechanisms thus leaving ssDNA gaps behind the fork that are not repaired until S phase is completed". Additionally, we have modified the text in "Results" to make clear that the formation of HR foci marks DNA repair.

3. Based on the analysis of Rad52 foci during S-phase in response to MMS treatment in checkpoint mutants, the authors conclude that the Mrc1-branch and Rad9-branch of replication checkpoints prevent recombinational repair during S-phase (Fig 6 and S4). Thus, the authors claim that the repair of ssDNA gaps by recombination is restricted to G2/M by the replication checkpoint. I found the data presented poorly convincing. First, some of the mutants studied present high level of spontaneous Rad52 foci, that renders kinetic studies difficult to interpret (Fig S4, sml1 rad53 chk1 and sml1 mec1 tel1).

We agree that the high level of spontaneous Rad52 foci in those triple mutants does not allow us to conclude that the sensors Mec1 and Tel1 and the effectors Tel1 and Chk1 have redundant functions. The text has been changed (see second paragraph in the Results section "*The replicative checkpoint prevents the assembly of MMS-induced HR repair centers during DNA replication"*).

Second, the accumulation of Rad52 foci during S-phase in the mrc1AQ rad9 double mutant, could be the consequence of the dynamic of DNA replication being affected by the loss of checkpoint function (more origins activated, more recombinogenic structures formed at replication forks..). Thus, I found difficult, based on the data provided, to conclude that the replicative checkpoint restricts the repair of ssDNA gaps to the G2/M phase. I would suggest the authors to provide stronger evidences to support their claim.

We agree that the accumulation of Rad52 foci during S phase in *mrc1*^{AQ} *rad9*Δ might be a consequence of defective replication dynamics by loss of checkpoint functions. We think that the best control to rule out this possibility is the analysis of MMS-induced Rad52 foci in rad53Δ and mec1Δ cells (Figure S5). These mutants are strongly affected in replication fork stability and dynamics (Berens & Toczyski, 2012; Branzei & Foiani, 2010). Despite these replication defects, rad53Δ and mec1Δ keep the regulation of HR in response to MMS (see that Rad52 foci appeared earlier at 0.01% than at 0.05% MMS and once cells reached G2/M). These arguments, together with the fact that Mrc1 is not required for the maintenance and restart of MMS-stressed forks (Tourrière *et al*, 2005), have been included in this revised text (see revised Sections in results and Discussion) to propose that the Mrc1 branch of the S phase checkpoint restricts HR repair during DDT to G2/M, a function that has been already shown for DSBs (Barlow & Rothstein, 2009; Alabert *et al*, 2009) and HU-stalled forks (Lisby *et al*, 2004; Meister *et al*, 2005; Barlow & Rothstein, 2009). Also note that an accumulation of recombinogenic structures at the fork would be a putative consequence of defects in this inhibitory function proposed for the replicative checkpoint.

Minor points

Page 10, lines 8: I don't think that "reverted" is the right word here. I would suggest "converted".

Changed as suggested.

Page 10, lines 22: "note that in the presence of MMS....of the Y-arc". What the authors meant here is unclear.

The difference in the Y-arc with and without MMS demonstrates that the lack of effect of MMS on the binding of Rad52-MN is not because the dose of MMS was insufficient to impair fork progression at the analyzed DNA fragment. This has been clarified.

Page 12, line 15: the reference to Meister et al. 2005 for the bud-to mother size ratio is meaningless.

Removed as requested.

Page 15, lines 25 'However, natural impediments are unlikely.....fork progression". What is the rational to claim this ?

The fact that fork progression is strongly impaired at that MMS dose. The sentence has been modified to make it clear.

Page 19, lines 12: refer to Figure S6 instead of S7.

Corrected.

Figure 5, panel C: the histogram on the top left is too small

Changed as suggested.

2nd Editorial Decision 06 March 2013

Thank you for submitting your revised manuscript for our consideration. Two of the original referees (comments copied below) have now reviewed it once more, and I am pleased to inform you that they are largely satisfied with the revisions. We shall therefore be happy to accept the paper for publication once the following minor/editorial points have been incorporated into the manuscript:

- Referee 2 maintains that there is still a strong emphasis on DDT that may not be fully justified, a concern already raised during the initial round of review. Therefore, please modify the manuscript text to a more cautious interpretation.

- Please add a brief 'Conflict of Interest' statement to the manuscript text, next to the acknowledgement and author contribution sections.

- Please combine all supplementary information into one single PDF of sufficient quality to facilitate download by our readers - keeping in mind that this part of the manuscript will not be type-set or copy-edited.

I am therefore returning the manuscript to you for one final round of minor revision, hoping you will be able to upload and re-submit the final corrected version as soon as possible. Should you have any questions in this regard, please do not hesitate to contact me directly.

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

"Rad51 replication fork recruitment is required for DNA damage tolerance" [manuscript # EMBOJ-2012-83909] revised

The authors addressed my previous concerns and added new experimentation and explanations, as well as the requested text changes. In particular, the authors can convincingly demonstrate the functionality of the MN fusions of Rad51 and Rad52 even under high MMS concentrations.

Referee #2 (Remarks to the Author):

The authors have successfully addressed most of my concerns. However, I am still concerned their emphasis on the DNA damage tolerance pathway in their introduction. They introduce PCNA modifications as well as ubiquitin and SUMO conjugation factors in great detail, without ever addressing their relevance for the observed phenomena experimentally. Hence, I would strongly recommend focussing the introduction on HR rather than damage tolerance and avoiding the term "DDT" or damage tolerance throughtout the manuscript. Instead, it would be more appropriate to use the term "replication fork escort" (according to reviewer no. 1) or "resistance to DNA damage". It would even be advisable to explicitly state that a currently there is no evidence for a connection to the "RAD6" pathway of damage tolerance.

2nd Revision - authors' response 08 March 2013

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The Introduction in the previous revised version was modified in order to focus more on homologous recombination (HR), as suggested by referee 2, since s/he was right in claiming that the manuscript does not address the connections between HR and the *RAD6* epistasis group of proteins. However, we think it is important to keep the current information about DDT for the following reasons:

The DNA damage response (DDR) includes DNA damage repair and DNA damage tolerance (DDT) mechanisms (the term “DNA damage resistance” is not employed to define DDR mechanisms, and “replication fork escort” refers only to one of the two cell-cycle regulated functions proposed for Rad52 and Rad51 in our work). HR proteins are involved not only in DSB-repair but also in DDT (reviewed in Friedberg, 2005; Heyer *et al*, 2010; Ciccia & Elledge, 2010). We have included these references in Introduction, together with the original observation by L. Prakash (Prakash, 1981), to make clear that we are not over-interpreting the role of HR proteins in DDT. In this work we have addressed the role of HR proteins in DDT. Therefore, it is important to introduce the general concept of DDT and its different mechanisms (translesion synthesis and template switching) before getting into a detailed introduction about HR, which cannot be understood without the previous concepts (for instance, the accumulation of ssDNA gaps and the loss of SCJs in *rad52*Δ and *rad18*Δ, two molecular intermediates that we follow in our work). The introduction about DDT needs also to include details about PCNA modifications not only to understand the mechanisms in which HR proteins might participate (in particular template switching), but also to underline that, despite the fact that HR proteins are required for DDT,

recombination is inhibited during replication (for instance, by mechanisms that require PCNA sumoylation). This is important because our results provide a scenario to explain this apparent contradiction, as discussed in "Discussion", where we propose that the replicative checkpoint inhibits the repair, but not the replicative functions of Rad52 and Rad51.

We disagree with the statement that there is no evidence for a connection between the *RAD52* and *RAD6* groups of proteins during DDT *("It would even be advisable to explicitly state that a currently there is no evidence for a connection to the "RAD6" pathway of damage tolerance"*). Different studies have recently shown their interconnection (Branzei *et al*, 2008; Minca & Kowalski, 2010; Vanoli *et al*, 2010). Actually, we have taken advantage of some of these common intermediates, *e.g.*, X-shaped molecules and ssDNA gaps, to address the functionality of the HR process. We are aware that there is some data that have been interpreted in terms of two independent pathways (Gangavarapu *et al*, 2007), indicating the complex genetic and molecular interactions between the different components of the error-free DDT mechanisms. In this frame, our results may help understand them in the future by solving important aspects about the mechanisms and regulation of HR proteins during DDT. We have included this general idea at the end of Discussion in order to reflect the right concern of the referee about these yet unclear interactions.

Branzei D, Vanoli F & Foiani M (2008) SUMOylation regulates Rad18-mediated template switch. *Nature* **456:** 915–920

Ciccia A & Elledge SJ (2010) The DNA Damage Response: Making It Safe to Play with Knives. *Molecular Cell* **40:** 179–204

Friedberg EC (2005) Suffering in silence: the tolerance of DNA damage. *Nat Rev Mol Cell Biol* **6:** 943–953

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Heyer W-D, Ehmsen KT & Liu J (2010) Regulation of Homologous Recombination in Eukaryotes. *Annu. Rev. Genet.* **44:** 113–139

Minca EC & Kowalski D (2010) Multiple Rad5 Activities Mediate Sister Chromatid Recombination to Bypass DNA Damage at Stalled Replication Forks. *Molecular Cell* **38:** 649–661

Prakash L (1981) Characterization of postreplication repair in Saccharomyces cerevisiae and effects of rad6, rad18, rev3 and rad52 mutations. *Mol. Gen. Genet.* **184:** 471–478

Vanoli F, Fumasoni M, Szakal B, Maloisel L & Branzei D (2010) Replication and Recombination Factors Contributing to Recombination-Dependent Bypass of DNA Lesions by Template Switch. *PLoS Genet* **6:** e1001205

