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Structure of the Rad50 DNA double-strand break repair protein in complex with DNA

Anna Rojowska, Katja Lammens, Florian Ulrich Seifert, Carolin Direnberger, Heidi Feldmann, Karl-Peter Hopfner

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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 27 May 2014

Thank you for submitting your manuscript to our editorial office. It has now been reviewed by three expert referees, who all consider the obtained structural basis of Rad50-DNA interactions of general importance and thus in principle suited for publication in The EMBO Journal. They nevertheless raise a number of major issues, especially regarding the functional significance of the structural observations, that would need to be adequately addressed before publication would be warranted.

I would therefore like to invite you to revise the manuscript according to the reviewers' suggestions, keeping in mind that our policy to allow only a single round of major revision makes it important to carefully answer to all points raised at this stage. In my view, of particular importance will be the additional follow-up on Rad50 mutation effects on DNA repair and end joining activities, as requested by referees 1 (major point 1) and 2 (points 2ii and 5). The other key point would be to better rationalize how ATP-dependent dimerization induces DNA binding (see referee 1 major point 3, referee 2 points 1 as well as 3, 4), which may be achieved by more in-depth comparative analyses of already available MR(N) complex structures in different states and conformations.

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; should you foresee a problem in meeting this three-month deadline, please let me know in advance and we could discuss the possibility of an extension.

Thank you again 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.

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REFEREE REPORTS:

Referee #1:

Rojowska et al present the X-ray structure of a minimal Rad50-Mre11 complex bound to a non-hydrolysable ATP analog, and DNA duplex. In addition, Hopfner and colleagues report mutagenesis of the Rad50-DNA interface to identify Rad50-DNA interaction motifs. Based on combined results from in vitro DNA interaction studies, and analyses of plasmid complementation studies of Rad50 deficient S. cerevisiae the authors propose roles for the Rad50 DNA interaction interface in telomere maintenance, but not DNA repair function in yeast. This Rad50-Mre11-DNA complex structure is a potentially pivotal advance contributing to our evolving understanding of MRX/N structure and biology, and therefore of general interest given that MRX/N is a central factor acting in the repair and signaling of DNA double strand breaks. Overall the manuscript is written and presented in a clear style. However, the conclusions drawn regarding the Rad50 separation of function mutations require further experimentation to be supported. Additional discussion of structural data would also help relate the current data to published literature.

Major points:

1. A central thesis put forth by the authors is that DNA binding to Rad50 is not germane to DNA double-strand break repair. This conclusion is based solely on qualitative survival spot dilution experiments using DNA damaging agents. In the text, the authors should better define the cellular effects of the agents chosen to analyze Rad50 DNA repair functions. Also, use of additional standard clastogen treatments that generate double strand breaks (eg. Ionizing Radiation, bleomycin) would bolster the authors claims that the DNA interaction surfaces are not important for DSBR. If these variants do indeed separate functions, can the authors elaborate on the observation that rad50-K103E R131E does appear sensitive to Camptothecin. Furthermore, quantitative survival assays should be used to allow for robust comparison between mutants.

2. On Pg6. the authors note comparisons of DNA bound and free forms show DNA binding induces small changes in Mre11-Rad50. This is illustrated in Fig. E2. However, structural alignments in Figure 5B appear to indicate larger changes are occurring in the coiled coils. A more detailed assessment and discussion of DNA induced conformational changes observed is merited. Do DNA-protein interactions to the coiled-coils account for scanning force microscopic evidence that Mre11-Rad50 complexes undergo large-scale conformational changes upon DNA binding (Moreno-Herrero, Nature 2005)? Are these motions due to crystal packing?

3. On page 10 the authors note: "Indeed, from the observed crystal structure it is not immediately obvious why DNA binding is functionally coupled to ATP- dependent dimer formation."

This statement is somewhat surprising given the depth of literature describing ATP-induced conformational changes in Rad50. Structural comparisons of the ATP-free and ATP bound Mre11-Rad50 surfaces, as well as the Rad50-Mre11-DNA complex reported here should be included along with a detailed analysis of the DNA binding surface changes in nucleotide free and bound forms. How do the Rad50 DNA binding surfaces compare to the Pyrococcus furiosus enzyme in AMP-PNP bound and unbound states? Is the conformation (not just primary sequence) of the DNA interaction surface conserved? Does DNA binding require motions of the Rad50 coiled-coils?

4. Can the authors comment on whether the Rad50 assembly that interacts with more than one DNA chain throughout the crystal lattice represent a possible mode of Mre11-Rad50 polymerization on duplex DNA, as is implied by chromatin immunoprecipitation experiments at defined DSBs in yeast.

Minor points

1. It is very difficult to see the structural diagrams in figure 3. These should be enlarged and more clearly labeled

2. Rad50 architecture is complex. A domain diagram in figure 1 showing Rad50 domain organization would help the non-expert reader.

3. The structural figures would benefit from more labeling - in particular the "SLH" motif should be identified in structural diagrams that refer to it in the text. Some relevant residues are also missing from the figures. For example, A111 is mentioned as interacting with DNA, yet it is not visible in any figure. Figure 2c would also be clearer if the legend described what the dots and dotted lines represented, as well as labeling of the "SLH" motif.

4. Pg.5 The authors mention the two complexes in the asymmetric unit are similar. A structural overlay should be provided. Is the asymmetry of DNA binding across the promoter conserved?

5. Pg. 14 paragraph 3. Typo: 8% Poliacrylamide

6. Pg. 14. What is the nature of the ramachandran outliers, and with what program were these statistics calculated?

7. Pg.7. Paragraph 1, sentence 1. AMPNP should be AMPPNP.

8.Pg 10. "These observations suggest that DNA is recognized in a more broad fashion by lobe I, presumably through the extensive positive electrostatic surface potential across both NBDs"
This sentence needs clarification.

Referee #2:

The Mre11-Rad50 complex plays a central role in sensing and repairing DNA double strand breaks via homology-directed repair and non-homologous end joining. ATP-driven conformation changes of Rad50 determine the choice of the repair pathways (Deshpande et al, 2014). One of the central questions is how the MR complex senses the damaged DNA. Both Rad50 and Mre11 independently bind DNA, and presumably each subunit may interact with DNA depending on the pathways. While the Mre11 - DNA complex structures have been reported and may provide some insights, it is unknown how Rad50 recognizes DNA. Previous studies reported that Rad50 interacts with DNA in an ATP-dependent manner (Hopfner et al 2000), although some groups reported that Rad50 also could interact DNA in an ATP-independent manner.
Here, Hopfner's group reported the long-awaited structure of Rad50-DNA complex in the presence of Mre11 peptide, AMPPNP and Mg^{2+}. In this structure, they observed that DNA binds between both coiled-coil domains of the Rad50 dimer. They identified that a strand-loop- helix motif on the NBD and part of the coiled coil near the NBD are important for the DNA binding by Rad50. They also propose that DNA binding to Rad50 is not critical for DNA double-strand break repair but is important for telomere maintenance using in vitro and in vivo experiments. The Rad50-DNA structure is clearly an important contribution to the field and allows the field to move step forward. In that sense, this work deserves publication in EMBO J. However, I do have some concerns and questions on this work which are not clear after carefully reading this manuscript. It would be very helpful for readers if authors address these issues and revise the current manuscript prior to publication.

1. P4 (line -5) Authors states that "The DNA binding, positively-charged groove between the coiled-coil is only formed after ATP driven dimerization of Rad50, showing how ATP binding triggers DNA binding". This sentence gives impression that the structure presented here provides a clue how ATP binding induces DNA binding by Rad50 dimer. However, in the text, the structure does not really provide such correlation. Authors may consider rewrite this sentence to avoid misleading.

2. P6 (line 7), "Amino acids K99, K108 and K109 are located on the top and outer face of the lobe I .. are positioned so that they may form additional interactions."

>> This is a very intriguing and perhaps important part.
(i) Although I can see the positions of each residues, it is difficult to see how far the residues are distant from the phosphate moiety. Authors may consider adding a mark (dot) for the interactions).

(ii) The mutational effects on K99, K108 are as significant as that of S768R or R765E, and these residues interact with the end of another (symmetrical-related) DNA. Thus, although Rad50 binds the central region of DNA, it also clearly interacts with DNA end. Furthermore, residues involved in these interactions are important in DNA-binding.
Deshpande et al (2014, EMBO J, Fig 5) demonstrated that Rad50 in the presence of ATP are crucial for DNA tethering. Amino acids K99, K108 and K109 could contribute DNA tethering and to understand the importance of these residues in DNA tethering, it would be very importance to perform in vitro NHEJ assay using these mutants as done by Paull's group. This Rad50-DNA structure may provide a clue for Rad50 mediated NHEJ.

3. P7, line -5; "We also mutated the residue R765 in the center of the Rad50 dimer cavity to check whether the DNA may reach the symmetry related binding site of the Rad50 dimer by traversing the positively-charged Rad50 groove."

>> Can author show a diagram for this or make a mark in the related figures ? How far would this residue be if DNA is modeled the symmetry related binding site of the Rad50 dimer ?

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5. Based on the mutational analysis (Figs 3 and 4), authors concluded that Rad50-DNA binding may not be involved in DNA repair. However this conclusion should be more carefully considered or reexamined for the following reason. Considering the fact that DNA binds broad surface area over the groove of the ATP-bound Rad50 dimer, it is possible that the single mutant of Rad50 may not affect the cell viability. In fact, although some mutant significantly diminished the DNA binding by Rad50, it is hard to conclude that the mutation resulted in null-mutant. Indeed, double mutation (K103ScE+R131ScE) clearly provided some impact on the viability, whereas single mutation did not. It is possible that even more multiple mutations may dramatically reduce the cell viability. Therefore, the authors should carefully reconsider their conclusion that the Rad50 - DNA binding is important for DNA repair.

6. Also, does Rad50 mutation affect the nuclease activity ? Although one may consider that the Rad50-DNA binding is a separate issue from the Mre11 nuclease activity, the Rad50 binding may facilitate the Mre11-DNA binding. The authors should at least discuss if Rad50-DNA binding may assist the Mre11 nuclease function (or show how Rad50 mutation affected Mre11 activity).

Minor errors:
1. First paragraph of result section: AMPPMP should be corrected.

2. P6, line 14: K115 is not conserved in PF Mre11. Or authors may define their conservation criteria.

3. SLH motif: what is the number for the strand and the helix ?

4. Fig 2A gives impression that the center of DNA to the Rad50 center, rather Rad50 tethers both ends of DNA.

5. P13: Was NCS applied during the refinement process at all ? If not, is there any reason for it ?

Referee #3:

Rojowska et al. report the structure of the Rad50 nucleotide-binding domain in complex with a small domain of Mre11 and DNA. This structure, being the first Rad50-DNA structure, is a interesting and important step is the structural characterization of the Mre11-Rad50-Nbs1 (MRN)

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complex. Although I am not in a position to judge the technical aspects of the crystallography, the conceptually importance of the structure and the well-executed genetic and biochemical validations of their conclusions will make this work of interest to a broad audience in the fields of DNA repair and recombination. Nonetheless, attention to the following minor issues would improve the paper.

The data in Figure 3 should be quantitated, the Hill equation should be fit to the data and apparent dissociation constants should be extracted.

Although I agree that "one key result of this work is that Rad50 does not appear to preferentially bind a DNA ends", I think that the authors should explicitly acknowledge that the fact that their structure contains only a fraction of MR and a pseudo-infinite DNA helix, with no end to bind, allows the possibility that Rad50 could bind ends, under other conditions.

MRN v. MRN/X v. MRX nomenclature should be consistent. I favor MRN, but switching back and forth is unnecessary and potentially confusing.

On page 7, "mutation of E798 to Q resulted in efficient dimer formation even in the presence of ATP" is unclear. It suggests that E798Q is also a dimer is the absence of ATP, which seems unlikely.

Page 7: "all other mutants displayed" -> "all other mutants examined displayed".

On page 7 it might be worth explicitly stating that PhiX174 RFII is a closed, double-stranded DNA molecule.

In Figure 4C, consistent length dashes should be used for the minus signs.

1st Revision - authors' response 03 September 2014

We thank all reviewers for their detailed and insightful evaluation and helpful comments on our manuscript. We are pleased that all three referees were positive with respect to the advancement this structure represents. Referee 1 says “This Rad50-Mre11-DNA complex structure is a potentially pivotal advance contributing to our evolving understanding of MRX/N structure and biology, and therefore of general interest”, referee 2 states “The Rad50-DNA structure is clearly an important contribution to the field and allows the field to move step forward. In that sense, this work deserves publication in EMBO J”, referee 3 says “This structure, being the first Rad50-DNA structure, is a interesting and important step is the structural characterization of the Mre11-Rad50-Nbs1 (MRN) complex. … the conceptually importance of the structure and the well-executed genetic and biochemical validations of their conclusions will make this work of interest to a broad audience in the fields of DNA repair and recombination.”
All referees had a number of points to improve the manuscript. We addressed all points and performed new experiments along their suggestions. In particular, we analyzed the effect of bleomycin and did an in vivo plasmid repair assay. These new data are added to the revised manuscript. We also substantially edited the manuscript, in particular the discussion, to address the referees’ comments. Please find below the point-by-point response. We think that the new data and editorial changes strongly improved the manuscript and we hope it can now be accepted for publication. Changes in the manuscript and the responses are highlighted in red (except some grammar corrections and corrected typos).

Referee #1:
Rojowska et al present the X-ray structure of a minimal Rad50-Mre11 complex bound to a non-hydrolysable ATP analog, and DNA duplex. In addition, Hopfner and colleagues report mutagenesis of the Rad50-DNA interface to identify Rad50-DNA interaction motifs. Based on combined results from in vitro DNA interaction studies, and analyses of plasmid complementation studies of Rad50 deficient S. cerevisiae the authors propose roles for the Rad50 DNA interaction interface in telomere maintenance, but not DNA repair function in yeast. This Rad50-Mre11-DNA complex structure is a potentially pivotal advance contributing to our evolving understanding of MRX/N

structure and biology, and therefore of general interest given that MRX/N is a central factor acting in the repair and signaling of DNA double strand breaks. Overall the manuscript is written and presented in a clear style. However, the conclusions drawn regarding the Rad50 separation of function mutations require further experimentation to be supported. Additional discussion of structural data would also help relate the current data to published literature.

Major points:
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We thank the referee for this comment and performed the yeast survival assay using the DNA double strand break inducing agent bleomycin (please see Figure 4 A). The results support the previous data using camptothecin and hydroxyurea. The weak effect of the double mutant Rad50-K103E R131E can be due to some growth defects since the mutant showed a somewhat delayed growth also on SDC-His and YPD plates without DNA double strand break inducing agents (see Fig 4A). For time reasons, we were unable to do quantitative survival curves, but we added a sentence saying that we do not want to rule out weak effects of the mutants also in repair activities.

2. On Pg6. the authors note comparisons of DNA bound and free forms show DNA binding induces small changes in Mre11-Rad50. This is illustrated in Fig. E2. However, structural alignments in Figure 5B appear to indicate larger changes are occurring in the coiled coils. A more detailed assessment and discussion of DNA induced conformational changes observed is merited. Do DNA-protein interactions to the coiled-coils account for scanning force microscopic evidence that Mre11-Rad50 complexes undergo large-scale conformational changes upon DNA binding (Moreno-Herrero, Nature 2005)? Are these motions due to crystal packing?

Again, we are grateful for this comment! We often see movements of the coiled-coil between different crystal forms. The coiled-coils are somewhat flexible and it is of course tempting to say that the contacts of coiled-coil lysines to DNA contribute, but we cannot rule out that the position of the coiled-coils is more influenced by crystal packing than by DNA. We shortened this part in "results" in favor of a more detailed explanation in the "discussion".

3. On page 10 the authors note: "Indeed, from the observed crystal structure it is not immediately obvious why DNA binding is functionally coupled to ATP- dependent dimer formation."
This statement is somewhat surprising given the depth of literature describing ATP-induced conformational changes in Rad50. Structural comparisons of the ATP-free and ATP bound Mre11-Rad50 surfaces, as well as the Rad50-Mre11-DNA complex reported here should be included along with a detailed analysis of the DNA binding surface changes in nucleotide free and bound forms. How do the Rad50 DNA binding surfaces compare to the Pyrococcus furiosus enzyme in AMP-PNP bound and unbound states? Is the conformation (not just primary sequence) of the DNA interaction surface conserved? Does DNA binding require motions of the Rad50 coiled-coils?

We rewrote this part to clarify the structural importance of ATP dependent Rad50 dimerization for DNA binding. Additionally we prepared an expanded view figure E7 to illustrate the arrangement of residues involved in DNA binding in the open nucleotide free and ATP bound closed form of the Mre11 Rad50 complex.

4. Can the authors comment on whether the Rad50 assembly that interacts with more than one DNA chain throughout the crystal lattice represents a possible mode of Mre11-Rad50 polymerization on duplex DNA, as is implied by chromatin immunoprecipitation experiments at defined DSBs in yeast.

This is an important observation and we also have looked at that. The Rad50 DNA oligomeric assembly as seen in the crystal structure would clash with the Mre11 nuclease domain (absent in our

structure) on the basis of structures that contained both Rad50 NBDs and full length Mre11. For that reason, we did not consider our Rad50 lattice contacts as relevant for MR oligomers. We addressed this comment in the “discussion”

Minor points
1. It is very difficult to see the structural diagrams in figure 3. These should be enlarged and more clearly labeled
We improved this figure.
2. Rad50 architecture is complex. A domain diagram in figure 1 showing Rad50 domain organization would help the non-expert reader.
We inserted a diagram to illustrate Rad50 domain organization and construct design.
3. The structural figures would benefit from more labeling - in particular the "SLH" motif should be identified in structural diagrams that refer to it in the text. Some relevant residues are also missing from the figures. For example, A111 is mentioned as interacting with DNA, yet it is not visible in any figure. Figure 2c would also be clearer if the legend described what the dots and dotted lines represented, as well as labeling of the "SLH" motif.
We improved this figure.
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See new Figure E2.
5. Pg. 14 paragraph 3. Typo: 8% Poliacrylamide
changed
6. Pg. 14. What is the nature of the ramachandran outliers, and with what program were these statistics calculated?
The outlier corresponds to residue N713 interacting with the Mre11 HLH motif and K115 involved in DNA binding. The values were calculated with the program Procheck.
7. Pg.7. Paragraph 1, sentence 1. AMPNP should be AMPPNP.
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8.Pg 10. "These observations suggest that DNA is recognized in a more broad fashion by lobe I, presumably through the extensive positive electrostatic surface potential across both NBDs"
This sentence needs clarification.
This part is rewritten

Referee #2:
The Mre11-Rad50 complex plays a central role in sensing and repairing DNA double strand breaks via homology-directed repair and non-homologous end joining. ATP-driven conformation changes of Rad50 determine the choice of the repair pathways (Deshpande et al, 2014). One of the central questions is how the MR complex senses the damaged DNA. Both Rad50 and Mre11 independently bind DNA, and presumably each subunit may interact with DNA depending on the pathways. While the Mre11 - DNA complex structures have been reported and may provide some insights, it is unknown how Rad50 recognizes DNA. Previous studies reported that Rad50 interacts with DNA in an ATP-dependent manner (Hopfner et al 2000), although some groups reported that Rad50 also could interact DNA in an ATP-independent manner.
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ATP binding induces DNA binding by Rad50 dimer. However, in the text, the structure does not really provide such correlation. Authors may consider rewrite this sentence to avoid misleading.

We removed this sentence in favor of a more balanced and expanded section in the discussion.

2. P6 (line 7), "Amino acids K99, K108 and K109 are located on the top and outer face of the lobe I .. are positioned so that they may form additional interactions."

corrected
>> This is a very intriguing and perhaps important part.
(i) Although I can see the positions of each residues, it is difficult to see how far the residues are distant from the phosphate moiety. Authors may consider adding a mark (dot) for the interactions).

We added the dots and thank the referee for this comment.

(ii) The mutational effects on K99, K108 are as significant as that of S768R or R765E, and these residues interact with the end of another (symmetrical-related) DNA. Thus, although Rad50 binds the central region of DNA, it also clearly interacts with DNA end. Furthermore, residues involved in these interactions are important in DNA-binding.
Deshpande et al (2014, EMBO J, Fig 5) demonstrated that Rad50 in the presence of ATP are crucial for DNA tethering. Amino acids K99, K108 and K109 could contribute DNA tethering and to understand the importance of these residues in DNA tethering, it would be very importance to perform in vitro NHEJ assay using these mutants as done by Paull's group. This Rad50-DNA structure may provide a clue for Rad50 mediated NHEJ.

This is an interesting suggestion. In Deshpande et al. the contribution of the Rad50 NBD domain construct with shortened coiled coil on DNA end tethering was very weak compared to the effects of the intact Mre11 Rad50 complex (see Fig. 5C in Deshpande et al. ; a 100 fold higher concentration of Rad50cd linked was used to observe equal effects for DNA end tethering compared to Mre11 Rad50). It was not possible for us in the time frame to transfer all mutants and establish expression and purification of full length TmMR. Therefore we decided
to analyze the influence of these amino acids on a yeast plasmid repair assay in vivo. Indeed two of the residues contacting the symmetry related DNA, K99 and K109 seem to be conserved in S. cerevisiae (Fig 2C) and the equivalent residues $K110^{Sc}$ and The 5^{Sc} have been mutated to glutamic acid. Whereas the ΔRad50 strain showed substantially reduced transformand yields for NcoI-linearized pRS315-Kan plasmids relative to supercoiled plasmid transformation, the Rad50 point mutants $K110E^{Sc}$ and $R125E^{Sc}$ led to only insignificant reduction in plasmid recovery (see Fig. E6). Although this is an interesting idea and hypothesis, our result currently do not support a significant involvement of these residues in non-homologous end joining processes in *S. cerevisiae.*

3. P7, line -5; "We also mutated the residue R765 in the center of the Rad50 dimer cavity to check whether the DNA may reach the symmetry related binding site of the Rad50 dimer by traversing the positively-charged Rad50 groove."
>> Can author show a diagram for this or make a mark in the related figures ? How far would this residue be if DNA is modeled the symmetry related binding site of the Rad50 dimer ?

The position of residue R765 is shown in Figure 3 and supplementary Figure E7. The residue is far away from any DNA in our crystal structure, but the access to the Rad50 groove is limited due to symmetry related molecules in the crystal packing.

4. It is not clear from this manuscript how widely the binding site of Rad50 surface covers. Is it possible that most of the regions in the surface over the Rad50 groove involved in DNA binding ? Based on the provided data, it seems highly possible. Authors should also use and cite the information for the DNA binding analysis of the groove/surface mutants of Rad50 reported by Lim et al (2011) and Mockel et al (2012). To provide an idea how far the residues (present and previously studied residues) are distant from the bound DNA, it would helpful to describe some distances (for some important residues, if not all binding residues).

We rewrote this part (see Page 11) to clarify the structural importance of ATP dependent Rad50 dimerization for DNA binding. Additionally we added the electrostatic surface potential in Fig. 5

and prepared an expanded view figure E7 to illustrate the arrangement of residues involved in DNA binding in the open nucleotide free and ATP bound closed form of the Mre11 Rad50 complex.

5. Based on the mutational analysis (Figs 3 and 4), authors concluded that Rad50-DNA binding may not be involved in DNA repair. However this conclusion should be more carefully considered or reexamined for the following reason. Considering the fact that DNA binds broad surface area over the groove of the ATP-bound Rad50 dimer, it is possible that the single mutant of Rad50 may not affect the cell viability. In fact, although some mutant significantly diminished the DNA binding by Rad50, it is hard to conclude that the mutation resulted in null-mutant. Indeed, double mutation (K103ScE+R131ScE) clearly provided some impact on the viability, whereas single mutation did not. It is possible that even more multiple mutations may dramatically reduce the cell viability. Therefore, the authors should carefully reconsider their conclusion that the Rad50 - DNA binding is important for DNA repair.

We thank the referee for pointing this out. We added this concern to the discussion.

6. Also, does Rad50 mutation affect the nuclease activity? Although one may consider that the Rad50-DNA binding is a separate issue from the Mre11 nuclease activity, the Rad50 binding may facilitate the Mre11-DNA binding. The authors should at least discuss if Rad50-DNA binding may assist the Mre11 nuclease function (or show how Rad50 mutation affected Mre11 activity).

At present, we have no data regarding the effect of Rad50 mutations on the Mre11 nuclease activity. We do not think that the nuclease activity is substantially compromised, otherwise one would expect stronger effects in the yeast survival assays, especially for camptotecin. We addressed this comment in the discussion.

Minor errors:
1. First paragraph of result section: AMPPMP should be corrected.
corrected

2. P6, line 14: K115 is not conserved in PF Mre11. Or authors may define their conservation criteria.
We thank the referee for pointing this out. A structural overlay indicated that residue K117 of PF Rad50 adopts an analogous position to residue K115 of TM Rad50 even though they are not aligned in the primary sequence. For that reason, we think the position of the charge is conserved in the 3D structures.

3. SLH motif: what is the number for the strand and the helix ?
We introduced a numbering system.

4. Fig 2A gives impression that the center of DNA to the Rad50 center, rather Rad50 tethers both ends of DNA.
Yes, it is in principle possible that Rad50 tethers two DNA molecules, however, we think this is due to lattice interactions. Referee 2 also noticed this and suggested an end joining assay. This we performed (in vivo plasmid ligation assay) and do not see a significant effect of mutations (K108 and K109). For this reason, we believe that this interaction rather represents internal DNA binding, mimicked by the quasicontinuous DNA in the lattice. The new data are added as Fig. E6 and the data are mentioned in “results” and in “discussion”

5. P13: Was NCS applied during the refinement process at all ? If not, is there any reason for it ?
We thank the referee for pointing this out. We indeed did not apply NCS because we had an excellent starting model for the $Rad50^{NBD}$-$Mre11^{HLH}$-ATPgS complex in the absence of DNA (1.9 Å PDB code 3QF7) and NCS tests did not improve R and R-free. For that reason, we decided to refine both molecules independently.

Referee #3:

Rojowska et al. report the structure of the Rad50 nucleotide-binding domain in complex with a small domain of Mre11 and DNA. This structure, being the first Rad50-DNA structure, is a

interesting and important step is the structural characterization of the Mre11-Rad50-Nbs1 (MRN) complex. Although I am not in a position to judge the technical aspects of the crystallography, the conceptually importance of the structure and the well-executed genetic and biochemical validations of their conclusions will make this work of interest to a broad audience in the fields of DNA repair and recombination. Nonetheless, attention to the following minor issues would improve the paper. The data in Figure 3 should be quantitated, the Hill equation should be fit to the data and apparent dissociation constants should be extracted.

We acknowledge the referees suggestion! However, after some initial test, we found it unrealistic to extract quantitative binding data and constants (Hill coefficient and Kd values) from the Rad50 EMSAs. As can be seen from the gel images, the shift pattern is very complex with multiple bands as well as "smears" and fits to hill equations did not produce meaningful values. To address the referee's point, we did two things. First, we included the protein concentration in the Fig. 3. Second, we quantitated the amount of free DNA and estimated the concentration where half of the free DNA is upshifted (Fig E4). These values are then shown in Fig. 3 as red lines. We hope this appropriately allows the reader to judge the effect of the mutations without overfitting the data.

Although I agree that "one key result of this work is that Rad50 does not appear to preferentially bind a DNA ends", I think that the authors should explicitly acknowledge that the fact that their structure contains only a fraction of MR and a pseudo-infinite DNA helix, with no end to bind, allows the possibility that Rad50 could bind ends, under other conditions.

We thank the referee for pointing this out. We only suggest that the observed DNA binding site on Rad50 (but not MR) has no direct preference of DNA ends, according to our structure. Of course it is still possible that the full MR complex directly binds DNA ends, perhaps via Mre11 or via a combined Mre11-Rad50 interface. We rewrote the discussion to include the referee's point.

MRN v. MRN/X v. MRX nomenclature should be consistent. I favor MRN, but switching back and forth is unnecessary and potentially confusing.
corrected

On page 7, "mutation of E798 to Q resulted in efficient dimer formation even in the presence of ATP" is unclear. It suggests that E798Q is also a dimer is the absence of ATP, which seems unlikely.
corrected

Page 7: "all other mutants displayed" -> "all other mutants examined displayed".
corrected

On page 7 it might be worth explicitly stating that PhiX174 RFII is a closed, double-stranded DNA molecule.
included

In Figure 4C, consistent length dashes should be used for the minus signs
corrected

Acceptance letter 25 September 2014

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

Referee #1

With the added data, discussion and analysis, Hopfner and colleagues have sufficiently addressed major and minor points in this revised manuscript. The structural, biochemical and genetic analysis

of Rad50-DNA complexes presented herein are indeed a significant advance.

Referee #2

In the revised text, Rojowska et al clearly resolved all the concerns this reviewer had. I believe that this well-written paper is an important and interesting piece of work, which will provide important insights into chromosome biology. Finally, I would like to ask authors to consider following points;

1. Page 5, r.m.s.d. for Ca atoms; 0.248 A and 0.354 A. How about to change 0.25 A and 0.35 A, respectively.
2. In the extended table 1, Rsym 68.6 (13.5) is odd. Please make sure they are reversed 13.5 (68.6). Also, authors used (2.7 A) for highest shell. Is it supposed to be the resolution range or a specific resolution ? If it is latter,are all the numbers in the parenthesis for this specific resolution, not the range (? ~ 2.7) ?

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