Manuscript EMBO-2015-92934

**Structural mechanism of ATP-dependent DNA binding and DNA end bridging by eukaryotic Rad50**

Florian Ulrich Seifert, Katja Lammens, Gabriele Stoehr, Brigitte Kessler, Karl-Peter Hopfner

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**Review timeline:**

- **Submission date:** 27 August 2015
- **Editorial Decision:** 28 September 2015
- **Revision received:** 10 January 2016
- **Accepted:** 13 January 2016

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Editor: Bernd Pulverer

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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.)

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1st Editorial Decision 28 September 2015

Thank you for submitting your manuscript on the eukaryotic Rad50 structure for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below.

While the two structure experts (ref 1, 2) support a revision, our expert biochemist (ref 3) is more critical. Overall, we have decided that we would like to invite a revision of this manuscript that addresses all the textural issues raised by referee 1-3. I will not outline these in detail here, but you will see that they range from the more cosmetic to important conclusions such as the conclusions about Rad50 DNA end binding (ref 3).

All three referees also raise issues with the description and indeed the analysis of a number of the Rad50 mutants described on p. 8-9, and we kindly request that these issues are addressed in detail. Briefly, ref 1 questions if S1205R prevents dimerization, and if K60E and R131E prevent DNA binding, while ref 2 requests data on the DNA binding and ATP hydrolysis of the R60 mutant, ref 3 requests evidence that the R60 mutant forms dimers in vivo.

Finally, referee 3 argues that a structure in the absence of Mre11 should be added to the paper. In our view, addition of such data will not be a precondition for publication in the EMBO Journal.

Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers as outlined above. I should add that it is EMBO Journal policy to allow only a single round of revision.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your 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 your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1:

The manuscript is clearly of a high scientific standard, and is generally clearly written and easy to follow. However, Figures 5 and 6, and the text describing them, need particular attention (see major comments below). Some other minor, mainly grammatical corrections are also listed. Some adjustments to figures, to improve clarity, are also suggested.

Major points:

Page 7 and Figure 5
Results / Structural Basis for ATP-dependent DNA binding by Rad50 and Details of ATP-dependent DNA binding of the Rad50 dimer

Here, the authors describe 4 DNA-binding motifs (I-IV); however, it is difficult for the reader to understand what these motifs actually are. Are they short sequences of amino acid residues, or just single amino acids? Also have these motifs been documented previously, if so, a citation is required. I strongly suggest including the identity of each motif in Figure 5, in order to aid the reader. I also suggest colouring the residues shown in Panel B, according to the motif they arise from. Figure 5 should also mark the central dyad, and indicate the 3'>5' and 5'>3' directionalities described in the main text (Page 7, Details of ATP-dependent DNA binding of the Rad50 dimer) in order to aid the reader.

Page 9
Results / Functional analysis of Rad50 DNA interactions in S.cerevisiae

The dimer interface mutant S1205>R in budding yeast Rad50 is described in the text. What is the evidence that this actually DOES prevents dimerization? Has this been reported elsewhere? If so, a citation is required. Similarly, K60>E, and K60E>E, R131>E mutations are described, as preventing DNA binding (by analogy to the Ct protein) - but have the effects of both mutations been formally been tested, anisotropy assay? Either in the Ct or Sc protein? I'm not sure it is just sufficient to state the expected outcome of each mutation, without formally demonstrating it. This needs addressing. Please also note that the introduced mutations are not lethal to budding yeast, as they all still grow under unstressed conditions - what they do is fail to rescue sensitivity to camptothecin treatment. Please rephrase accordingly.

Page 10 / Figure 6

Why the odd oligonucleotide numbering system? Please remove or make more systematic.

Please put the +/- ATP 35mer DNA binding data (Supplementary Fig S6) into Figure 6; Make this panel (A). The comparison of binding to oligo 3.1 (5-base 5'overhang) and oligo 4.1 (5 base-pair 3' overhang) should be directly overlaid on the blunt-ended 17mer (oligo 2.1) to aid the reader, Make this panel (B). The remaining curves (oligos 3 and 4) should go on a third plot (C), along with oligo 1 (30bp dsDNA) as a reference.
There appears to be negative anisotropy for the 35bp ssDNA (13), grey boxes, why?

Please also separate the keys, and place them with the relevant isotherms. Label as (B) and (C). Also, please report determined Kds on the figure itself, this will aid the reader. The 'DNA-diagrams' used in the supplementary figures, would also be very useful in this figure.

Why are the anisotropy data fitted to a single-site binding model with ligand depletion? Is this actually the most appropriate model to use? Especially as the binding model does not properly fit the data for binding to the ssDNA or 5' overhang containing DNA. These are both sigmoidal, and not rectangular hyperbola. Please amend and comment.

Minor points:

Page 2, Introduction
"... can result in loss or alteration of the genetic information". Remove 'the'.

Page 3, Introduction
"The choice between NHEJ and HR is regulated in a cell-cycle dependent manner". True, but NHEJ can occur in both G1 and G2, whereas HR is restricted to G2, due to the availability of a sister chromatid. Suggest a rephrase here.

Page 3, Introduction
"The nuclease activity of MRN helps to remove Ku from DNA ends,". Please see recent paper from the Jackson laboratory, about neddylation/ubiquitination of Ku [doi: 10.1016/j.celrep.2015.03.058] - consider rephrasing/rewriting.

Page 5, Results
"mutation in this domain T841->K in human) was found in a patient..." Where is the location of this mutation in the CtMre11RBD? In the non-visible C-terminal polypeptide chain? Or in helix a5? - this requires some clarification.

Page 7, Results
"and we determined the structure by molecular replacement using CtRad50NBD as search model". Amend to read:
"determined the structure by molecular replacement using CtRad50NBD as a search model".

Page 7, Results
"partially degraded the DNA during the relatively long crystallization time (4 months)". Remove the word 'relatively'.

Page 7, Results
"For the further discussion, we will denote..." Amend to read:
"For subsequent discussion, we will denote..."

Figure 3, Panel A.
The green 'cross-links' are not visible enough. A change of colour may be more suitable.

Figure 3, Panel C
Please use the same # of crosslinks range in both plots (i.e. 1 to 3).

Figure 7, Panel B.
Red and Yellow circles (interactions with Rad50) should be defined in the figure legend.

Referee #2:
Seifert et al present new X-ray crystal structures of eukaryotic Mre11-Rad50 and Rad50-DNA
complexes. This significant advance by the Hofpner group expands our understanding of the molecular mechanism of the DNA double-strand break repair mechanism initiated by the conserved Mre11-Rad50 complex. In particular, this work documents two important developments. First it describes the first eukaryotic Rad50 structures that in addition to revealing the added structural complexity of eukaryotic homologs, also provides an expanded testable basis for understanding the ATP-regulated DNA binding activity of Rad50. Secondly, this work extends our understanding of the molecular assembly of the eukaryotic Mre11-Rad50 complex beyond the published work on archaeal and eubacterial MR complexes to reveal an extended Mre11 helical bundle that tethers to the Rad50 coiled-coil. Overall this generally well presented work has important implications for our understanding of MRN regulated DSB repair, and gives new insight into Mre11 inactivation in Ataxia Telangiectasia Like Disorder (ATLD). Added discussion and experimentation would help clarify the authors model and results.

Major points

1. Structure determination: As presented, the details of the Rad50-DNA complex structure determination are somewhat unclear. In the results (pg.7) it is stated that due to ambiguity and possible nucleolytic degradation the authors did not model a defined sequence. Given the extended crystallization period, and the fact that in general the functional interactions do not involve base-specific DNA interactions this cautious approach seems reasonable. However supplementary figure S7c shows modeled base pairs. Were the nucleotide bases modeled at all? More details and improved continuity of the presentation are required to better document how the refinement was completed.

2. The results and discussion of the yeast genetics is under developed. CPT does not directly cause double strand breaks. This should be clarified in the text. The discussion of "lethality" of the mutants is also not clear. The mutants are not lethal. They confer sensitivity to CPT, but do not appear lethal, so the discussion should appropriately address this. Also, do the mutations confer sensitivity to DSB producing agents (e.g. bleomycin or IR.

3. Does the motif 3 (S. cerevisiae R60E mutation) or the equivalent mutant in the C. Thermophilum protein impact ATP stimulated DNA binding? Does it impact ATP hydrolysis or DNA-stimulated ATP hydrolysis. While the functional data in yeast suggest this mutation is important for CPT survival, its impacts on Rad50 function in vitro do not appear to be documented. This should be tested to substantiate the authors conclusions.

4. Page. 6 - model and Figure 3D. Is it possible to model conformational states measured with Small angle X-ray scattering data of the ATP-gamma-S bound and unbound states by ab initio reconstruction, or to validate the ATP-bound Mre11-Rad50 with the molecular models for the eukaryotic Mre11/Rad50 built in this study? Further, does the SAXS data confirm that in the absence of nucleotide that the eukaryotic Mre11/Rad50 head adopts a highly extended conformation similar to that described for the eubacterial Mre11/Rad50?

Minor points:

5. The presence of internal cavities in archaeal Rad50 has been shown to influence ATP regulated conformational states. These conformations are also associated with detailed salt bridging networks that change upon binding of nucleotide. While only the nucleotide bound state is documented is this work, can the authors compare structural and sequence conservation of the salt bridging and core cavity networks in the eukaryotic protein compared to the published P. furiosus MR complex. In the context of DNA bound ligand, such analysis might point to possible mechanisms of DNA-regulated (stimulated) ATP hydrolysis that has been observed for the baker's yeast MRX complex.

6. Motif IV appears to coincide with the "signature coupling helices" described by Tainer and colleagues (NSMB 2011). This linkage of the DNA binding site to the coiled-coils and Mre11 binding site could provide a conduit for interesting coupling of ATP binding to the coiled-coils, and Mre11. On a related point, the DNA-free and DNA bound states are compared in figure S4F, but these differences are not discussed. Do the structural comparisons glean insights into possible crosstalk between DNA binding site and coiled-coil conformations?
7. Where is the T481K ATLD5/6 mutation expected to be located in human Mre11? Can this mutation and its potential impacts be modeled and commented on further. Also, mutagenesis studies in fission yeast based on archaeal structures have been published. It would be informative to discuss where these mutations in fission yeast Rad50 that impact S. pombe DSB repair in map in this new eukaryotic structure.

8. Can the authors further discuss on the impacts of Rad50S mutations in insertions I and II? Possibly these mutations could be marked in the sequence alignment and a diagram made for the structural roles for these positions in the C. thermophilum protein.

9. Figure 3a - label relevant protein landmarks - ie N and C-termini of Rad50 and Mre11 if possible to clarify this figure.

10. Figure S2. It is hard to see the white text on black background.

11. Is there any evidence that the C. Thermophilum MRN complex is thermostable?

12. Figure 1B. Some of the stereo labels overlap with the structure and are difficult to read. E.g. Signature S1208 ...

13. Figure 5 - better contrasting colors for the Protomer A and Protomer B would help this figure. Also, coloring the N- and C-terminal ABC ATPase lobes would be informative.

Referee #3:

In this manuscript the authors present the crystal structures of the ATPgammaS bound nucleotide binding domain of Rad50 from a thermophilic eukaryote in complex with either DNA of the Rad50 binding domain of Mre11. These structures will serve as an important starting point for experiments that will address the mechanism through which the thermophilic Rad50/Mre11 complex is involved in DNA metabolism. Given the limited number of experiments presented that test predictions from the model and the ambiguity in interpreting the results from the DNA binding experiments, the study is too preliminary for publication in EMBO J.

The authors are miss using the term 'tethering' of DNA end. What they are addressing with their structure can best be classified as end bridging; two ends of a broken DNA held in close proximity presumably in a relative rigid/static structure. Tethering implies a flexible link and I believe this was first coined in the context of a model where the flexible arms of the Rad50 protein molecules, bound to different DNAs, interacted in a dynamic fashion. The terminology should be adjusted through the manuscript.

The crystals from which the model Rad50 DNA binding is derived were grown in the presence of Mre11. This is a bit sloppy. It is unclear how the presence of this Rad50 binding protein influences the model. It seems to me that we can have more confidence in the model when Mre11 is left out of the crystallization conditions.

The experiment that tests whether Rad50 mutants are lethal due to compromised DNA binding or dimerization is weak. It is based on the assumption that Arg61 mutant still forms dimers in vivo, but this is not demonstrated experimentally.

The authors interpret the fluorescence anisotropy experiments to measure DNA affinity as indicating that the Rad50 dimer has a preference for binding DNA ends with annealed 3’ overhangs. I think this conclusion is not valid based on the presented experiments. It is based on the observation that DNA with annealed 5’ overhangs binds with less affinity and explained by the notion that in case of 5’ overhangs the nicks would be at the interface of DNA and protein, while for 3’ overhangs the nicks would be at the solvent side. This all depends on the length of the overhangs. It is the length and not the polarity of the overhang that would dictate the affinity.
Referee #1:

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We revised Fig. 5 along the referee’s suggestion. We added the strand polarities, dyad, motifs and color. We also denoted R1204 as motif V and added to the text “The five DNA-binding motifs are short sequence regions or single amino acids and bind in total 12 bases within the 18 bp duplex”. We thank the referee for pointing this out and hope the revised figure and text adds the requested clarity.

Page 9

Results / Functional analysis of Rad50 DNA interactions in S.cerevisae

The dimer interface mutant S1205>R in budding yeast Rad50 is described in the text. What is the evidence that this actually DOES prevents dimerization? Has this been reported elsewhere? If so a citation is required. Similarly, K60>E, and K60E>E, R131>E mutations are described, as preventing DNA binding (by analogy to the Ct protein) - but have the effects of both mutations been formally been tested, anisotropy assay? Either in the Ct or Sc protein? I'm not sure it is just sufficient to state the expected outcome of each mutation, without formally demonstrating it. This needs addressing.

Please also note that the introduced mutations are not lethal to budding yeast, as they all still grow under unstressed conditions - what they do is fail to rescue sensitivity to camptothecin treatment. Please rephrase accordingly.

The referee is correct that we incorrectly used the term “lethality” and we corrected this to “CPT sensitivity”. We also performed the requested biochemical assays. We purified Rad50 signature motif and DNA binding site mutants and tested them in dimerization and DNA binding assays. We find that all mutants except the signature motif mutant form dimers in the presence of ATP, confirming our model. Furthermore, the DNA binding site mutants show substantially decreased affinities for DNA in the fluorescence anisotropy experiments, confirming the structural data. These new data add important controls and we thank the referee for the suggestions! We added these data to Fig. EV3 (dimerization) and Fig. 6D (DNA binding) and revised the corresponding text (page 9).

Page 10 / Figure 6

Why the odd oligonucleotide numbering system? Please remove or make more systematic.

We deleted the numbering.
Please put the +/- ATP 35mer DNA binding data (Supplementary Fig S6) into Figure 6; Make this panel (A). The comparison of binding to oligo 3.1 (5-base 5’overhang) and oligo 4.1 (5 base-pair 3’ overhang) should be directly overlaid on the blunt-ended 17mer (oligo 2.1) to aid the reader. Make this panel (B). The remaining curves (oligos 3 and 4) should go on a third plot (C), along with oligo 1 (30bp dsDNA) as a reference.

We added the mutant data and rearranged the data along the referee’s suggestions.

There appears to be negative anisotropy for the 35bp ssDNA (13), grey boxes, why?

We thank the referee for pointing this out. If the angle between the absorption and emission transition is higher than 54.7° the anisotropy becomes negative (Lakowicz, J.R. Fluorescence polarization. In: Principles of Fluorescence Spectroscopy. Plenum Press. New York. London. 2006, 353–382. http://dx.doi.org/10.1007/978-0-387-46312-4_10). However, we don’t know why negative anisotropy appears specifically with ssDNA and only at low protein concentrations. A study with YFP has seen negative anisotropy and attributed it to effects caused by dimerization coupled to unidirectional Förster transfer (Shi X, Basran J, Seward HE, Childs W, Bagshaw CR, Boxer SG. Anomalous Negative Fluorescence Anisotropy in Yellow Fluorescent Protein (YFP 10C): Quantitative Analysis of FRET in YFP Dimers. Biochemistry. 2007;46(50):14403-14417. doi:10.1021/bi701575n.). Given that ssDNA might form transient interactions, possibly a similar phenomenon caused by DNA-DNA interactions happens for our ssDNA.

Please also separate the keys, and place them with the relevant isotherms. Label as (B) and (C). Also, please report determined Kds on the figure itself, this will aid the reader. The ‘DNA-diagrams’ used in the supplementary figures, would also be very useful in this figure.

Changed as suggested

Why are the anisotropy data fitted to a single-site binding model with ligand depletion? Is this actually the most appropriate model to use? Especially as the binding model does not properly fit the data for binding to the ssDNA or 5’ overhang containing DNA. These are both sigmoidal, and not rectangular hyperbola. Please amend and comment.

We thank the referee for pointing this out. Since Rad50 forms dimers and since we titrate the protein there could be an increased formation of Rad50 dimers with higher protein concentrations, which would lead to a sigmoidal type of binding curve. For that reason, we reanalyzed the data with a Hill model, which leads to an arguably better fit of the data. The Hill coefficients typically range from around 1 for the higher affinity binding DNA molecules to around 2 for the lower affinity molecules (Table S2). Original analyses are still reported in the appendix.

Minor points:

Page 2, Introduction
"... can result in loss or alteration of the genetic information". Remove ‘the’.

corrected

Page 3, Introduction
"The choice between NHEJ and HR is regulated in a cell-cycle dependant manner". True, but NHEJ can occur in both G1 and G2, whereas HR is restricted to G2, due to the availability of a sister chromatid. Suggest a rephrase here.

We thank the referee for the comment, left the cell cycle preference out and rephrased to: “The choice between NHEJ or HR is regulated by the cell through multiple mechanisms including the initiation of end resection and chromatin composition”

Page3, Introduction
"The nuclease activity of MRN helps to remove Ku from DNA ends, ".
Please see recent paper from the Jackson laboratory, about neddylation/ubiquitination of Ku [doi: 10.1016/j.celrep.2015.03.058] - consider rephrasing/rewriting.

We rephrased to “The nuclease activity of MRN helps to clear blocked or modified DNA ends and generates initial...”.

Page 5, Results
"mutation in this domain T841->K (in human) was found in a patient..."
Where is the location of this mutation in the CtMre11RBD? In the non-visible C-terminal polypeptide chain? Or in helix a5? - this requires some clarification.

We added the corresponding residue in the text and highlighted it in Fig. 1.

Page 7, Results
"and we determined the structure by molecular replacement using CtRad50NBD as search model". Amend to read: "determined the structure by molecular replacement using CtRad50NBD as a search model".

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Amend to read: "For subsequent discussion, we will denote...

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Figure 3, Panel A.
The green 'cross-links' are not visible enough. A change of colour may be more suitable.

Changed to a darker green with thicker lines.

Figure 3, Panel C
Please use the same # of crosslinks range in both plots (i.e. 1 to 3).

Changed as suggested

Figure 7, Panel B.
Red and Yellow circles (interactions with Rad50) should be defined in the figure legend.

We defined the circles (key interacting DNA phosphates).

Referee #2:

Seifert et al present new X-ray crystal structures of eukaryotic Mre11-Rad50 and Rad50-DNA complexes. This significant advance by the Hofpner group expands our understanding of the molecular mechanism of the DNA double-strand break repair mechanism initiated by the conserved Mre11-Rad50 complex. In particular, this work documents two important developments. First it describes the first eukaryotic Rad50 structures that in addition to revealing the added structural complexity of eukaryotic homologs, also provides an expanded testable basis for understanding the ATP-regulated DNA binding activity of Rad50. Secondly, this work extends our understanding of the molecular assembly of the eukaryotic Mre11-Rad50 complex beyond the published work on
archaeal and eubacterial MR complexes to reveal an extended Mre11 helical bundle that tethers to the Rad50 coiled-coil. Overall this generally well presented work has important implications for our understanding of MRN regulated DSB repair, and gives new insight into Mre11 inactivation in Ataxia Telangiectasia Like Disorder (ATLD). Added discussion and experimentation would help clarify the authors model and results.

Major points

1. Structure determination: As presented, the details of the Rad50-DNA complex structure determination are somewhat unclear. In the results (pg. 7) it is stated that due to ambiguity and possible nucleolytic degradation the authors did not model a defined sequence. Given the extended crystallization period, and the fact that in general the functional interactions do not involve base-specific DNA interactions this cautious approach seems reasonable. However supplementary figure S7c shows modeled base pairs. Were the nucleotide bases modeled at all? More details and improved continuity of the presentation are required to better document how the refinement was completed.

We clearly see density for the base pairs and added a generic sequence (G:C) for the refinement to also maintain a correct stereochemistry. Adding bases improved both density and statistics. However, we could not assign the sequence of the DNA to the model because of the ambiguity. We revised the text to:

“The asymmetric unit accommodates only 15 of the 22 base pairs, suggesting that either Mre11 in the crystallization drops partially degraded the DNA during the long crystallization time (4 months) or the DNA molecules are shifted between adjacent asymmetric units. For that reason, we are unable to assign the sequence of the co-crystallized DNA to the DNA model. However, density for the DNA backbone and bases is for the most part well defined and we consequently modeled bases using a generic oligo(dG:dC) 15mer into the electron density, which improved the stereochemistry, R-values and the DNA density.”

2. The results and discussion of the yeast genetics is under developed. CPT does not directly cause double strand breaks. This should be clarified in the text. The discussion of “lethality” of the mutants is also not clear. The mutants are not lethal. They confer sensitivity to CPT, but do not appear lethal, so the discussion should appropriately address this. Also, do the mutations confer sensitivity to DSB producing agents (e.g. bleomycin or IR.

The referee is correct and we thank the referee for pointing these semantic mistakes out. We changed “lethality” to “CTP sensitivity” and revised the text to “CTP-induced DNA damage”. We tested S1205R, E1235Q and R1201E in a prior study where we found also sensitivity to HU and bleomycin (Rojowska et al., EMBO J 2014). The effect of R1201E in that study suggests that the DNA binding site of Rad50 as identified in the current study is critical for the repair of HU and bleomycin induced DNA damage. We added a sentence in the discussion to address this question by the referee.

3. Does the motif 3 (S. cerevisiae R60E mutation) or the equivalent mutant in the C. Thermophilum protein impact ATP stimulated DNA binding? Does it impact ATP hydrolysis or DNA-stimulated ATP hydrolysis. While the functional data in yeast suggest this mutation is important for CPT survival, its impacts on Rad50 function in vitro do not appear to be documented. This should be tested to substantiate the authors conclusions.

We now experimentally tested the R60E mutation along with some other mutations in the DNA binding assays and observe a substantial reduction in ATP stimulated DNA binding affinity. These data corroborate our structure and we thank the referee for suggesting this experiment. With respect to the ATPase, we find that Rad50 NBD by itself has very little ATPase activity even in the presence of DNA and might require Mre11 and/or the coiled-coils for full activity. For that reason, we have concentrated on the DNA binding experiments in this study and reserved ATPase assays for future work.
4. Page 6 - model and Figure 3D. Is it possible to model conformational states measured with Small angle X-ray scattering data of the ATP-gamma-S bound and unbound states by ab initio reconstruction, or to validate the ATP-bound Mre11-Rad50 with the molecular models for the eukaryotic Mre11/Rad50 built in this study? Further, does the SAXS data confirm that in the absence of nucleotide that the eukaryotic Mre11/Rad50 head adopts a highly extended conformation similar to that described for the eubacterial Mre11/Rad50?

We originally attempted modeling but the obtained models were not conclusive. For that reason we decided not to include any models of the complex on the basis of the SAXS data. Presumably we have different subpopulations in the presence of ATP, preventing us to obtain a single, conclusive model. We also do not see a highly extended state in the absence of ATP as in the case of the bacterial protein. It is therefore likely that the eukaryotic protein is not stabilized in an open conformation as seen with the bacterial protein. This is all we can safely say for now. We suggest remaining on the conservative side and only report on the basis of the scattering curves and p(R) that the complex adopts a more compact state or a population of compact states in the presence of ATP without overinterpreting the data. To address this comment, we revised this section (p6).

Minor points:
5. The presence of internal cavities in archaeal Rad50 has been shown to influence ATP regulated conformational states. These conformations are also associated with detailed salt bridging networks that change upon binding of nucleotide. While only the nucleotide bound state is documented in this work, can the authors compare structural and sequence conservation of the salt bridging and core cavity networks in the eukaryotic protein compared to the published P. furiosus MR complex. In the context of DNA bound ligand, such analysis might point to possible mechanisms of DNA-regulated (stimulated) ATP hydrolysis that has been observed for the baker’s yeast MRX complex.

We thank the referee for pointing this out. Indeed, like the P. furiosus protein, CtRad50 possesses a variety of cavities and salt bridges between both lobes. Hence, we fully agree with the referee that a similar mechanism could be in place than has been characterized with the P. furiosus protein. The Chaetomium and Pyrococcus Rad50 are, however, quite distinct in details. Since we have only one nucleotide state, we are not in a position to discuss conformational changes and alternating hydrogen-bonding networks. We added a couple of sentences to the discussion to address this point. In fact, our new DNA binding data with the signature motif mutant suggest an ATP induced conformational change within the CtRad50NBD.

6. Motif IV appears to coincide with the "signature coupling helices" described by Tainer and colleagues (NSMB 2011). This linkage of the DNA binding site to the coiled-coils and Mre11 binding site could provide a conduit for interesting coupling of ATP binding to the coiled-coils, and Mre11. On a related point, the DNA-free and DNA bound states are compared in figure S4F, but these differences are not discussed. Do the structural comparisons glean insights into possible crosstalk between DNA binding site and coiled-coil conformations?

This is an interesting question. We do not see much difference between the DNA bound and free form of Rad50-ATPγS in the globular part although we do see a shift in the coiled-coils. It appears that the conformations of the coiled-coils is at least partially stabilized by the crystal lattice. Furthermore in one Rad50 structure (no DNA) we have additionally Mre11 bound, which could also influence the structure of the coiled-coil. At present, we do not see how DNA would directly influence the coiled-coil, but do not want to rule out such an effect in solution (and not the crystal lattice) either.

7. Where is the T481K ATLD5/6 mutation expected to be located in human Mre11? Can this mutation and its potential impacts be modeled and commented on further. Also, mutagenesis studies in fission yeast based on archaeal structures have been published. It would be informative to discuss where these mutations in fission yeast Rad50 that impact S. pombe DSB repair in map in this new eukaryotic structure.

We added the mutation to Fig. 1 and Fig EV1. It is likely, that the mutation affects the stability of the RBD and the interaction between the RDB and Rad50 coiled-coil. We added this comment to the revised manuscript.
8. Can the authors further discuss on the impacts of Rad50S mutations in insertions I and II? Possibly these mutations could be marked in the sequence alignment and a diagram made for the structural roles for these positions in the C. thermophilum protein.

We added these mutations in the sequence alignment and also added a figure to the Appendix (Appendix Fig S2) as requested. Most of the Rad50S mutations map to the surface, so we still think they are involved in a macromolecular interaction site. The two mutations in or near insertions I and II, however, map to the Rad5-ATP dimer interface. Based on the structure, we think these mutations would impact on ATP-dependent Rad50 dimer formation or ATP hydrolysis. We added a sentence to the results part.

9. Figure 3a - label relevant protein land marks - ie N and C-termini of Rad50 and Mre11 if possible to clarify this figure.

We added the termini as well as labels for the proteins and the coiled-coils.

10. Figure S2. It is hard to see the white text on black background.

We changed the font to bold, increasing the readability.

11. Is there any evidence that the C. Thermophilum MRN complex is thermostable?

We did not formally explore the thermostability of CtRad50 or CtMre11 with precise measurements, but our observations with proteins from Chaetomium indicate that thermostability is only moderately increased compared to other eukaryotic proteins and certainly not near the thermostability of proteins from hyperthermophilic archaea.

12. Figure 1B. Some of the stereo lables overlap with the structure and are difficult to read. E.g. Signature S1208 ...

We corrected this in a revised figure.

13. Figure 5 - better contrasting colors for the Protomer A and Protomer B would help this figure. Also, coloring the N- and C-terminal ABC ATPase lobes would be informative.

We changed lobe II to brown which increased the contrast to the yellow/beige of the other Rad50 protomer and also allows distinction between lobes I and II. We also added labels “lobe I” and “lobe II”.

Referee #3:

In this manuscript the authors present the crystal structures of the ATPgammaS bound nucleotide binding domain of Rad50 from a thermophilic eukaryote in complex with either DNA of the Rad50 binding domain of Mre11. These structures will serve as an important starting point for experiments that will address the mechanism through which the thermophilic Rad50/Mre11 complex is involved in DNA metabolism. Given the limited number of experiments presented that test predictions from the model and the ambiguity in interpreting the results from the DNA binding experiments, the study is too preliminary for publication in EMBO J.

The authors are miss using the term 'tethering' of DNA end. What they are addressing with their structure can best be classified as end bridging; two ends of a broken DNA held in close proximity presumably in a relative rigid/static structure. Tethering implies a flexible link and I believe this was first coined in the context of a model where the flexible arms of the Rad50 protein molecules, bound to different DNAs, interacted in a dynamic fashion. The terminology should be adjusted through the manuscript.

As suggested, we changed “tethering” to “bridging”.
The crystals from which the model Rad50 DNA binding is derived were grown in the presence of Mre11. This is a bit sloppy. It is unclear how the presence of this Rad50 binding protein influences the model. It seems to me that we can have more confidence in the model when Mre11 is left out of the crystallization conditions.

The presence of Mre11 in the crystallization drops is not expected to influence the structure of Rad50 in the crystal. By interacting with Rad50 in solution Mre11 could influence the kinetics of Rad50 crystal formation, but since it is not part of the crystals, by thermodynamic reasoning Mre11 does not influence the conformation of Rad50 in the crystal itself. I hope this explains the concern by the referee.

The experiment that tests whether Rad50 mutants are lethal due to compromised DNA binding or dimerization is weak. It is based on the assumption that Arg61 mutant still forms dimers in vivo, but this is not demonstrated experimentally.

We now added three lines of experiments to address the referees point. We first measured dimerization of Rad50 mutants in vitro and find that Rad50^{NBD}-R61E can dimerize in the presence of ATP like the wtRad50^{NBD}. We also tested DNA binding by this mutant and find, as predicted by the structure, that DNA binding affinity is severely compromised. Finally, we also tested the integrity of the complex in yeast extracts by gel filtration and Western blot analysis. We find that S. cerevisiae MRX K60E R131E (the equivalent of C. thermophilum R61E) forms large complexes that migrate similar to the wild type complex, demonstrating that the overall integrity of the complex for the DNA binding site mutants is preserved. We hope that the combination of these three experiments satisfactorily shows that the observed effects on CPT sensitivity by K60E or the K60E R131E double mutant is not due to a dimerization defect but rather due to the structurally predicted and experimentally tested corrupted DNA binding. All these new data are added to the manuscript (Fig. 6, Fig. EV3, Fig. S4B).

The authors interpret the fluorescence anisotropy experiments to measure DNA affinity as indicating that the Rad50 dimer has a preference for binding DNA ends with annealed 3’ overhangs. I think this conclusion is not valid based on the presented experiments. It is based on the observation that DNA with annealed 5’ overhangs bind with less affinity and explained by the notion that in case of 5’ overhangs the nicks would be at the interface of DNA and protein, while for 3’ overhangs the nicks would be at the solvent side. This all depends on the length of the overhangs. It is the length and not the polarity of the overhang that would dictate the affinity.

We thank the referee for this comment. We clearly find a difference between the two types of short overhangs, so this needs to be explained. For short complementary 5’ overhangs, we see only a modest effect compared to non-complementary overhangs or blunt ends, while 3’ overhangs lead a substantially increased affinity, similar to “continuous” DNA. The structure offers a simple and plausible explanation for this observation as depicted in Fig. 7. Of course, if one would test longer complementary overhangs, one might find different effects, as correctly pointed out by the referee. However, we based our analysis on the observation that micro-homologies are typically short and in the range of 1-5 basepairs in vivo. We hope this explanation convinces the referee that our model is of relevance for this physiologically important range, although a more detailed analysis including longer overhangs would certainly be of interest for future studies. To address the referee’s point we included a sentence in the discussion: “Along the same arguments, the preference for 3’ overhangs might be alleviated for longer complementary overhangs.”

I am very pleased to accept you paper for publication in the EMBO Journal. All three referees came back with positive verdicts and rank the work highly.

In our view the Title and Abstract are well framed. It may be worth adding minimal detail on the biochemical data in the abstract, as this is referred to in a very general manner at present. It may also be worth to include a simplified form of the last sentence of the discussion ‘They provide the first structural framework for the ATP regulated interaction of a chromosome associated ABC ATPase of the Rad50/SMC/RecN protein family with DNA.’
We are also satisfied with the author checklist as completed - please ensure that the Antibody sources noted on the list are also recorded within the paper.

A few minor issues should be addressed:
1) We did not find a 'conflict of interest' section - this is mandatory (even if there is none to declare - see guide to authors)
2) The manuscripts refers to fig EV6, which we could not locate (legend or figure)
3) Please assemble a text for the synopsis, which can include 4-5 bullet points that summarize salient findings of the paper in a concise manner that is largely non-redundant with the abstract. We will include a small image that visually illustrates the findings best. Since this has to work as an icon size, we would suggest fig 3A, left hand side, but if you prefer the model with the full complex please let us know.

Please complete the relevant forms listed below - my colleagues will be in touch for any additional information required.

We aim to publish this paper fast track online and will enter it into our dedicated fast track production modus. Please ensure that all the relevant information is provided to avoid subsequent delays. The reason for this is a related study which was submitted independently and published a short time ago in this journal (30.12.2015; The EMBO Journal (2015) embj.201592462; DOI 10.15252/embj.201592462). Since this study was published during re-review, we would ask to refer to it briefly at the end of the paper as a 'note added in proof'.

We are about to mandate that corresponding author have an ORCID record - please generate one if you have not done so already (it takes <90 seconds).

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Referee #1:

The authors have satisfactorily dealt with each issue raised by referees. I am happy that this paper is now suitable for publication in The EMBO Journal.

Referee #2:

Hopfner and colleagues have suitably addressed referee comments.

Referee #3:

The authors have addressed my comments in a satisfactory manner.
Please follow the journal's authorship guidelines in preparing your manuscript.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where relevant:

### A. Figures

#### 1. Data

The data shown in figures should satisfy the following conditions:

- The data were obtained and presented according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- Figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- Graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- If n=5, the individual data points from each experiment should be plotted and any statistical test employed should be justified.
- Source data should be included to report the data underlying graphs. Please follow the guidelines set out in the authorship guidelines on Data Presentation.

#### 2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- A specification of the experimental system investigated (e.g. cell line, species name).
- The assay(s) and method(s) used to carry out the reported observations and measurements.
- An explicit mention of the biological and chemical entity(ies) that are being measured.
- An explicit mention of the biological and chemical entity(ies) that are alternated/variable in a controlled manner.
- The exact sample size (n) for each experimental group/condition, given as a number, not a range.
- A description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- A statement of how many times the experiment shown was independently replicated in the laboratory.

Definitions of statistical methods and measures:

- Common tests, such as t-test (please specify whether paired or unpaired), simple t-tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section.
- Are tests one-sided or two-sided?
- Are there adjustments for multiple comparisons?
- Exact statistical test results, e.g., P values = x but not P values < x;
- Definition of "center values", as median or average;
- Definition of error bars as S.D. or S.E.M.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

### B. Statistics and general methods

1. a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?

2. b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.

3. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?

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5. a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.

6. b. For animal studies, include a statement about blinding even if no blinding was done.

7. For every figure, are statistical tests justified as appropriate?

### C. Reagents

Please fill out these boxes. Do not worry if you cannot see all your text once you press return.

**USEFUL LINKS FOR COMPLETING THIS FORM**

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- http://www.ega.ebi.ac.uk
- http://jjj.biochem.sun.ac.za
- http://www.bioevidences.net/
- http://bioinformatics.net/whatis/
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D- Animal Models

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., AntibodyMap (see link list at top right), IDGewevo (see link list at top right).

7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.

9. For experiments involving live variates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.

10. We recommend consulting the ARRIVE guidelines (see link list at top right) to ensure that all relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) for non-English recommendations. Please confirm compliance.

E- Human Subjects

12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.

13. For publication of patient photos, include a statement confirming that consent to publish was obtained.

14. Report any restrictions on the availability (and/or on the way) of human data or samples.

15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.

16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.

17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Coordinates and structure factors have been deposited in the Protein Data Bank under accession codes 5A9F for CMA vs11BD-Re600BD and 5D4C for CMA vs10BD-DNA.

F- Data Accessibility

19. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'.

20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as EGA or JWS Online.

G- Dual use research of concern

21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section.

22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SMAL, CaML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIWAM guidelines (see link list at top right) and deposit their model in a public database such as BioModels database (see link list at top right) or JWS Online. If applicable, if a computer software or source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.