THE EMBO JOURNAL

Manuscript EMBO-2014-88299

DNA End Recognition By The Mre11 Nuclease Dimer: Insights Into Resection And Repair Of Damaged DNA

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Review timeline:

Submission date:	21 February 2014
Editorial Decision:	19 March 2014
Revision received:	29 June 2014
Editorial Decision:	17 July 2014
Revision received:	20 July 2014
Accepted:	21 July 2014

Editor: Hartmut Vodermaier

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 19 March 2014

Thank you for submitting your manuscript on Mre11-DNA complex structure for our editorial consideration. We have now received the comments of three expert referees, who all find the determination of longer duplex DNA being bound by Mre11 dimers interesting and potentially important for our functional/physiological understanding of MR complex roles. However, all three referees also raise a substantial number of overlapping major concerns with the study, which in its present form does not appear to be ready for publication. Some of these points are requests for further experimentation and control data, in particular for the biochemical and genetic follow-up experiments. However, the majority of issues refer to major concerns with the data presentation and interpretation; these problems appear to require re-analyses of many of the data, as well as substantial reorganization and rewriting of all sections of the manuscript.

Given that these major issues appear to be in principle addressable, I would like to give you the opportunity to revise and resubmit an improved manuscript for our further consideration. I should however stress that it will be essential to thoroughly re-work the whole analysis and presentation, taking into account in particular the detailed suggestions and requests of referee 2. It will also be important to improve the mutational validation work, including ruling out confounding effects on MR complex assembly in the tested mutants. When rewriting the manuscript, please try to make use of the extended format of EMBO Journal articles to clearly describe background and data and lay out the underlying concepts in a generally accessible manner; I would also appreciate if you would closely consult with colleagues who are native speakers of English for editing the manuscript text. Finally, it would be good if the derived concepts and conclusions could be graphically illustrated also in a summary model figure (again, please note that we have no strict limit on figure numbers).

It is our policy to allow only a single round of major revision (three months), and 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 us know in advance to discuss the possibility of an extension, as it will be very important to fully answer all points of the referees during this one round. Also, please do not hesitate to contact me in case you should have any additional question regarding this decision or the reports.

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

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REFEREE REPORTS:

Referee #1:

The manuscript from Cho and colleagues addresses the current model of DNA recognition by the Mre11 nuclease dimer. Previous reports suggested each Mre11 subunit that recognizes a DNA duplex helps to bridge two DNA ends. The authors have used longer DNA molecules that they feel reflect more accurately the damaged DNA in physiological conditions and propose alternative models of how Mre11 dimers can recognize DNA ends. They present the crystal structure of Mre11 from Methanocaldococcus jannaschi bound to DNA molecules of 17 or 22 bp with 4-5 nt overhang. They showed that one Mre11 dimer binds to one, rather than two DNA molecules, and suggest that DNA bridging by Mre11 can occur by oligomerization of Mre11 dimers. They describe the potential role of the Mre11 dimeric interface in end resection, by introducing a disulphide bond which changes the confirmation of Mre11 and enhances the nuclease activity, while conversely disturbing the dimeric interface decreases the nuclease activity.

To understand the physiological importance of the proposed Mre11-DNA structure they introduced mutations to Mre11 that disrupt the Mre11-DNA interface in biochemical assays and in yeast. Interestingly, they showed that these mutants were less effective in non-homologous end joining by an HO endonuclease-induced DSB. Using two different mutations, they saw a very different sensitivity to CPT, as the mutant K184A was very sensitive to both DNA damaging agents - CPT and PHL, but the mutant K62A was only sensitive to PHL. They proposed that Top1-DNA links need an additional confirmation that is achieved by interaction of Mre11 and Xrs2, as an additional inactivation of Mre11 binding site to Nbs1 resulted high sensitivity to CPT. An alternative model for previously published data about how Mre11 is interacting with DNA is proposed and may provide a more complete explanation of how the Mre11 dimer is involved in DNA metabolism.

Overall the data presented is of interest to the field and could shed new light on the mechanism by which Mre11, and the complex as a whole, affect DNA metabolism. The paper is quite difficult to read, even for someone in the field (as is often the case for structure papers) and the authors could take some steps to more clearly present the data and help a general audience understand the experiments and conclusions. In addition, quantification of any of the biochemical assays is strangely absent, as are important controls for the yeast data. Until some of these major issues are addressed, I remain intrigued but remain somewhat skeptical of some of the major conclusions.

1. The authors inconsistently use confusing terminology in the opening paragraphs of the results without clearly defining them. "The B-form DNA, subunit A and B, strand A and B, B-type DNA" etc. I assume B-type and B-form DNA refer to the same thing. Maybe consider using some other abbreviations so it is always clear whether you are talking about a DNA structure, one of 2 DNA strands or one of 2 dimer subunits.

2. In general the paper is very confusing to follow given the number of mutations and the differences in residue number between organisms. Providing a diagram/summary of the location of the mutations on the subunit, the relative class of mutation (dimer interface, DNA contacts, capping domain, nuclease etc) and its equivalent place in the organisms used would be very beneficial to the reader in relating the figures and experiments. Combining a simplified version of Supplemental

figure 3 with the mutations generated for example.

3. In figure 3A, it would be helpful to point out the region and residues described in the text more clearly. Similarly, the mutations made in 3E-G could be more clearly indicated in the structure. This would be more relevant to me than the 2 figure panels dedicated to the mononucleotide binding.

4. The relevance of the mononucleotide binding was unclear to me. Is this artifactual from the crystallization process or is there something more important that can be concluded? The argument is made that these longer DNAs are more physiologically relevant but in the context of a chromosome, I am not sure if these contacts would be relevant or whether they are simply specific to the DNA structures and process used.

5. In figure 3E-G, figure 4 E-J and figure 5 A-C the authors compare mutated forms of Mre11 for activity on a number of synthetic substrates. Although conclusions are made in the text regarding relative activity, no quantification of the expected bands is provided to allow comparisons between experiments. It is also concluded that exo and endo activities are affected but how one can discern that from the gels (which products are indicative of either activity) is not immediately clear.

6. In the same figures, no evidence that the proteins are added in equivalent amounts is presented.

7. In figure 4, it would be helpful to the reader if the structural features described, namely the capping domain and four helix bundle, were clearly indicated.

8. In figure 4, is there evidence that these mutations affect the rotation of the capping domain or is this assumed based on the structure?

9. In figure 5, it is unclear why the experiments are now done in the context of Rad50 rather than with Mre11 alone as in all previous experiments. It makes it somewhat difficult to compare.

10. A major issue to consider in the interpretation of the yeast data is the status of the MRX complex. Previous conclusions regarding nuclease mutations were later recanted as the mutations disrupted the whole complex creating essentially a null situation. The authors should provide evidence that the interactions with Rad50/Xrs2 are not disrupted as the interpretation of the data hinges on knowing this information.

Referee #2:

General comment:
Sung et al. present the structure of M. jannaschii Mre11 (MjMre11) in complex with DNA. Mre11 is a nuclease and central player in DNA repair and homologous recombination. As they discuss, previously published structures of P. furiosus Mre11 in complex with smaller DNA fragments do not fully explain how Mre11 recognizes and processes DNA. Sung's et al. MjMre11-DNA structure is different from these previous structures, as it contains a longer DNA molecule bound across the Mre11 dimer. Sung et al. are thus able to identify new DNA-binding residues in Mre11 and test their role in DNA processing using in vitro nuclease assays. They also mutate related residues in yeast Mre11 and analyze their role in resistance to genotoxic agents and in end-joining.
The MjMre11-DNA structure in principle represents considerable progress towards a better understanding of Mre11 and would of central interest to the readers of EMBO J and a general audience. Nevertheless there are serious limitations in the current manuscript that need to be improved, e.g. DNA is bound at some considerable distance to the Mre11 active site(s)) that need to be discussed more thoroughly by the authors. In addition, some biochemical and biological experiments need to be repeated. Inconsistencies need to be clarified.
While the MjMre11-DNA structure is a new highlight in the field of DNA repair, its current presentation needs to be substantially revised. It unfortunately includes inconsistent argumentation, gross grammatical errors and word usage errors even on the level of scientific terms (such as the use

of "subunits" for NCS-related molecules in the asymmetric unit). While some parts such as the introduction would better be written in a more concise way the level of detail on the methods is insufficient. Figures could be improved using consistent color schemes and different viewing angles and the sequence alignment also needs substantial corrections. Although it sounds harsh, the authors are strongly advised to substantially rewrite and correct their manuscript, so that the MjMre11-DNA structure is presented in a way it deserves. It would be a pity if the general readership was not able to appreciate this important structure due to an inaccessible description.

Abstract:
1) Please mention the source organism, M. jannaschii.
2) '....using longer DNA molecules that more accurately reflect damaged DNA,..'. Perhaps change to "....using longer DNA molecules that more accurately reflect a broken chromosome"

Introduction:
3) I suggest to rename the Mre11 complex to Mre11-Rad50 complex.
4) The three different models (and figure 1) should be left out or their origin needs to be clarified.

Results:
Overall structure of the DNA bound MjMre11 complex:
5) The DNA substrates used for crystallization could be named DNA1 and DNA2 to make things clearer.

6) Supplementary Table 1:
The overall statistics are fine, but there are some issues and some missing details:
- The units for r.m.s. deviation of bond angles should be degrees ({degree sign}), not ≈.
- It appears that the Ramachandran plot was analysed using Procheck. As Procheck is outdated, it would be preferable to use Molprobity. It would also be recommendable to reference the program used for validation (Procheck, Molprobity or anything else).
- The table footnotes define the r.m.s. deviation in B factors between bonded atoms. However, there seems to be no such entry in the table itself. Please amend.
- Please also list I/ .
- Given the low resolution of the structure, it would be helpful to list the resolution at which I/ {less than or equal to} 2 and Rsym {greater than or equal to} 50 %.
- Please state average B-factors for protein, nucleotide and DNA atoms.
- Please correct typographical errors such as "relections", "generouslly" etc.

7) The authors do not allow the reader to make any judgment of the quality of the structure. Given the low resolution, it would be helpful to provide examples of electron density (omit maps, 2Fo-Fc maps) for important parts of the Mre11 protein (e.g. the Mg site and DNA interacting loops), for bound DNA and for the bound nucleotides.

8) The authors claim there are almost no differences between the MDB1 and MDB2 co-structures, "except a few additional DNA contacts with Mre11 in Mre11-MB2". This phrase is intriguing.
- What are these differences? How could they come about? Could they tell us something about different binding states?
- Could the authors present an superposition of these two structures
- In that context, only one Mre11 dimer out of three in the a.u. seems to have bound DNA. Is it possible that the DNA molecule mediates crystal contacts?
- In the latter case, can the authors exclude that the differences in DNA contacts are a function of slightly different crystal packing?

9) Sung et al. compare their structure only to the so-called synaptic DNA-Mre11 complex published by Williams et al 2008. However, Williams et al. also reported a second DNA-Mre11 complex, a branched DNA complex, which the authors chose to ignore completely. How does the DNA complex solved by Sung et al. compare to this second complex? In that context, the authors should rethink the three models they postulate in the introduction and may have to rewrite their discussion and structural comparisons.

10) Materials and methods:
- Protein Expression and Purification: The paragraph lacks important information, such as primer

sequences used for cloning, expression conditions, cell disruption, buffer compositions and purification (type of columns etc.). Please correct.
- Structure determination and refinement: The authors state that both crystal forms contain one dsDNA-MjMre11 complex in the a.u. However, according to the results part, an a.u. contains three Mre11 dimers and one DNA. Please correct.
- During refinement, NCS restrains were applied. Was NCS strictly enforced, even in the case of the DNA-bound Mre11 dimer?

11) Figure 2A and B
- The imposition of DNA from the PfMre11-DNA structure should have been presented in a separate figure to avoid confusion.

12) Figure 3 A-D
- Most of the structural figures are inconsistent when it comes to color schemes and different viewing angles. Further, close-up figures and better labelling (active site etc.) would also be helpful for the general readers.

DNA recognition by Mre11:
13) Supplementary Figure 3:
The Mre11 sequence alignment needs to be corrected:
- The color scheme seems to be misleading. Yellow is supposed to indicate "strictly conserved residues", according to the figure legend. However, unless the authors define strict conservation in some rather unusual way (which they should then state), it is not conceivable how a residue such as K273 merits the classification "strictly conserved". According to the alignment, K273 is an N, L or K in other organisms, i.e. it is highly variable. Similar cases are N75 (S or A in other Mre11), E245 (R in all other organisms), R247 (otherwise F), F277 (otherwise R) and many more. The same goes for "similar residues" colored in green: The only similarity at position 133 is that K, W and R are all amino acids. Other examples are positions 196 (N, S, Q, K) and 253 (N, K, I, S, V). Please redo the labelling of similar and strictly conserved residues in your alignment, perhaps using programs such as Clustal Omega and Jalview.
- It is surprising that, in the case of human Mre11 (hMre11), the authors obviously used a sequence downloaded from the PDB. Thus, the sequence includes a hexahistidine-tag and several residues "X", which presumably indicate selenomethionines in the hMre11 structure. Please use the native hMre11 sequence as available on Uniprot or GenBank.
- In case of SpMre11, it seems unlikely that the first residue is an A. Please adjust the sequence numbering (or the sequence).
- According to the legend, nuclease motifs are highlighted using brown. It may be a printer/screen problem, but these boxes appear rather to be red.
- In the legend, please define the sequences, referencing the source organism and a UniProt or NCBI (or similar) accession number (e.g "MjMre11, M. jannaschii, UniProt accession number XXXXXX").

14) Page 6, last paragraph:
- The authors mention that residues such as Y13, R55 and R89 can be imposed on PfMre11 structures and refer to Fig 3A and SI Fig3. However, these figures do not show a structural alignment although such a structural superimposition would be useful and should be added by the authors (also for MjMre11 vs hMRe11 and vs yeast Mre11).

Additional interactions between MjMre11 and Nucleotides:
15) Page 6:
- 'We also observed three mononucleotides bound to an MjMre11 subunit other than the DNA-binding units'. The word "subunit" does not seem to be the right term and is actually confusing. The authors seem to refer to the three Mre11 dimers in the asymmetric unit, and they should put it this way.
- The authors should show the binding sites of these nucleotides in an overview figure to make it easier to

16) An interesting finding is that nucleotides are bound by non-DNA-binding Mre11 dimers. The authors postulate that these nucleotides may be derived from the co-crystallized DNA. However, the authors should consider following scenarios and should test them:

- The authors may well be right and the nucleotides are derived from the DNA ligands. The authors could test this hypothesis by analyzing the DNA before and after incubation with MjMre11 and Mg2+.
- The nucleotides may be contaminants introduced when adding DNA for co-crystallization. Did the authors purify their DNA samples, e.g. using HPLC or PAGE?
- The nucleotides may have been co-purified from E. coli.
- In any case, the authors may be able to identify the nature of these nucleotides using methods such as mass spectrometry or NMR.
- Unfortunately, the authors don't discuss these nucleotides any further. They, in contrast to the bound DNA ligands, may yield clues about the biochemical mechanism of Mre11, as one of them is actually close to the active site.

Mutational analyses:
17) The authors claim that Arg89 is a conserved residue involved in DNA-binding. If compared to the multiple sequence alignment (Suppl. figure 3), Arg89 is absolutely not conserved (nor similar) to the other Mre11 homologs, which harbor either a Q, S o T residue in the same position. Intriguingly, the authors generated an R89S mutant, which showed no more nuclease activity. The authors gloss over the fact, however, that SpMre11 and ScMre11 retain their nuclease activity despite having an S in exactly the same position. Please discuss.

18) Materials and methods:
- A 'DNA oligonucleotides and P32 labelling' chapter should be included, containing information about DNA sequences, labelling - and annealing procedure. The DAR134 substrate seems to contain two different hairpin conformations (double bands), and hence an additional purification step is required. The purification can easily be performed by separating the annealed substrates on a 10 % native PAGE, followed by gel excising and elution (diffusion) into H20. Also, could the samples have been separated on a 20% sequencing gel to obtain a better resolution? Information regarding the detection method is also missing.
- The sizes of the nucleolytic degradation products are labeled in all figures, however, the size marker itself is not shown. Please include the size marker, at least in form of a supplementary figure.
- The authors have not included any information about sequences of the quick change primer nor the site-directed mutagenesis protocol. Please provide this information.

19) Figure 3 E-G

- A small part of the gel (above the substrate bands) should be shown, to get an idea of the purity of the various DNA substrates. This is true for all the nuclease assay gels.
- It would also be informative to include quantitative analysis of the substrate degradation using ImageQuant etc. for all nuclease assays.
- Fig 3F: As mentioned earlier, the DAR134 substrate needs to be purified to avoid the two different hairpin conformations.
- Fig. 3G: The figure has a very low resolution and should be optimized in order to draw any conclusions. It seems that less substrate could have been added in the rxn's containing K129A and K132D, and if so the activity of the Lys mutants would be more similar to wt.

20) Supplementary fig. 5A
- The band shifts are stacked in the wells and have not migrated into the gel. Why haven't the P32-labeled substrates been used and detected by phosphor imaging, as for the nuclease assays? Could the complexes have been separated on a lower percentage native PAGE or an agarose gel in order to avoid the well shifts? Also, DNA binding could have been verified by Biacore or filter binding assays to detect binding affinities of dynamic DNA- protein complexes.

21) Lys132 is a recurring theme in this paper, partly because it "is close to DNA" and partly because it is deemed a "conserved basic residue". According to the alignment in suppl. Figure 3, this residue is a tryptophan in PfMre11, which seems to contradict somewhat the notion of conservedness. Also, the authors should make their case for a role in DNA processing more plausible. What is the exact orientation of Lys132 towards DNA? What kind of interaction do they expect? All that information cannot be derived from the figures.

Quaternary structural changes of Mre11:

22) Page 8, 2nd paragraph.
- 'To examine conformational changes of Mre11, we compared the structures of five MjMre11 dimers (three dimers in this study; one DNA-free MjMre11, PDB 3AUZ; one MjMre11 bound to Rad50, PDB 3AV0; Lim et al, 2011).' The paragraph is somewhat confusing. Is only the alignment of MjMre11-DNA towards DNA-free MjMre11 (PDB 3AUZ) shown in figure 4A, or is the MjMre11-Rad50 (PDB 3AVO) also included in the structural comparison? Information about the different structures (and PDB codes) should be included in the figure legend.

Cross-linking of a dimeric interface increases the nuclease activity:
23) Figure 4:
- It is not clear which samples were under reducing or non-reducing conditions. Please add a label or a description mention in the figure legend.
- Fig. 4F: As for figure 3F, the DAR134 substrate needs to be purified prior to nuclease activity measurements.
- Fig. 4G: As for figure 3G, the assay has too low resolution and the amount of substrate added to each reaction does not seem to be consistent.
- Fig. 4I: Even though the inactivity of the I302Y mutant is obvious (at least when comparing the amount of substrate), the DNA substrate (control) is degraded or contaminated, and consequently the experiments should be repeated using a purified substrate.
- Fig. 4J:

24) Page 9, 1st paragraph:
- 'Importantly, this mutant showed comparable or even increased activity relative to the wild-type Mre11 in an oxidized state, suggesting that alteration of the subunit arrangement of an Mre11 dimer could regulate the nuclease activity (Figure 4E-G, lane 12 and 13).'
It should be mentioned that di-sulphide bonds are formed during oxidizing conditions (and consequently a stable Mre11 dimer), to make it easier for the reader to understand the conclusion.

Mre11 Dimerization is critical for nuclease activity:
25) Figure 5:
- Please provide information about protein concentrations, as well as labels of the different DNA substrates.
- The authors do not provide any details about the protein expression and purification of MjRad50.
- All the figures have a very low resolution, and thus the authors should be cautious drawing too many conclusions. Eg: For the DAR134 substrate it is clear that the dimeric mutant actually has a nuclease activity, at least when compared to the control sample. However, the nucleolytic degradation pattern differs when comparing the dimeric mutant to wt.
- 'Although it is not completely abrogated, the "dimeric" mutant showed significantly decreased exonuclease activity compared to wild type MjMR toward TP124/580 in the absence and presence of ATP (or ATP S) (Figure 5A, lane 4-7).' The only significant reduction in endonuclease activity can be seen for APO (Lanes 2 and 3), and not for ATP/ATprS as stated by the authors.
- Fig. 5C: The resolution of the image is far too low, and the result should be optimized prior to publication.

In vivo analysis:
26) It should be clear which yeast strains (genetic background) have been used in the different in vivo assays.
27) Figure 6 B: please correct the labeling of the axis to the correct percentage or does the wildtype has only 0.3 percent survival in Figure 6B ?
28) Figure 6D
- The spot assays on the lower part of the figure must have been incubated for a shorter period of time if compared to the spot assays in the upper panel. This is easily observed when comparing the phenotypes of sgs1 (1) with sgs1 (6). Thus, the authors are strongly encouraged to repeat the spot assays, in which all the stains should be spotted on the same plates and tested towards both CPT and phleomycin. There is no need to spot the same strains twice as seen for both nr (1) and (6), and (5) and (7), respectively. Further, the number of parallels should also be mentioned in the material and methods.
- It is not clear whether mre11 sgs1 (2) is an mre11 deletion mutant or not. Please label properly.

29) Figure 6 E-F: Please comment

30) Page 10, 3rd paragraph:
- ".....", we deleted SGS1, the gene required for one of the two.....". Please be more specific, both when it comes to the protein the gene codes for and the resection pathway.

Discussion:
31) The authors claim that they disrupted the MjMre11 dimer interface by introducing "bulky hydrophobic residues". However, the triple mutant they used introduces only one bulky hydrophobic residue, but one alanine and one arginine. Please correct.

Referee #3:

In this manuscript the authors describe a new X-ray crystallography structure of Mre11 bound to DNA. Mre11 is a component of the Mre11-Rad50 complex that has diverse roles in DNA break repair. The DNA binding and nuclease activities of Mre11 are crucial aspects of its function. A structure of Mre11 bound to DNA exists from previous X-ray crystallography studies. However that complex was formed with short oligonucleotide DNA molecules and two such oligos were bound to the Mre11 dimer. In that work speculation about the role of the protein in DNA end joining, based on the juxtaposition of DNA ends in this structure, was presented. Here an Mre11 dimer was crystalized in the presence of a longer DNA molecules that bind as one continuous strand across the protein dimer. New amino acids involved in DNA binding were identified and their importance tested by mutations and activity assays. The structure presented here is very likely closer to an authentic Mre11-DNA complex as it would be involved in DNA end processing and repair. Although an initial report always gets more attention, this structure deserves equivalent attention as it is likely more informative and will be useful for stimulating further studies for Mre11 function. However the current manuscript is not in optimal form to present this information clearly or in the best context for general appreciation. The description of the structures is difficult to follow, some data in the figures does not add to understanding and some data in the supplement seems much more important. Possibly intriguing results are not discussed much and seemingly less interesting or confusing results are present in more detail. The English is sloppy and in some instances outright confusing.
Here a list of specific points to address:

1. It would be interesting to compare the DNA-protein interface identified here with other DNA-protein interfaces, particularly others where the DNA is similarly distorted.

2. It would be interesting to report more information and better describe the three different dimer forms in the asymmetric unit. The nucleotide bound to two of these is mentioned but in a confusing manner. This data may be important in its own right. More detail on this aspect would be interesting. The protein-protein interfaces may be a result of the crystallization but they may also indicate possibly important interfaces. The structural description part of the paper would be stronger with these elements expanded. In general a clearer coherent description of the structural elements revealed here would help.

3. The description of the DNA fragment bound across Mre11 is very confusing. Referring to right and left halves of the DNA is not helpful or even accurate. I do not know what this means. Referring to halves of the DNA is not useful. Referring to the different strands of the double-stranded DNA as "a" and "b" and to the two Mre11 protein subunits of the dimer are "A" and "B" is not at all a good choice. It would be useful to reconsider the wording and describe what is important and different about the one continuous DNA strand bound across the active site compared to the previous structure which included short oligos that could not bind across the whole dimer.

4. Reference to base positions with 3 letter abbreviations, p. 5 Cyt, Thy, Gua, is non-standard and confusing. Similar structural work usually refers to bases with one letter and a number indicating position along the DNA strand sometimes with or without a ' to indicate different strands.

5. The structural bits shown in some figures are hard to understand. For instance in figures 3 B,C and D, and 4 B and D, it is very difficult to figure out where these parts of the structure fit into the whole. Better figures and description are needed.

6. Figure 1 is not necessary and not informative. Where as figure S1 is in my opinion much more useful information and would be appreciated in the main paper.

7. The mutations made in the newly discovered DNA binding site are tested for DNA binding by an activity assay. Presumably if DNA is not bound then it cannot be acted upon by the protein it does not bind to. The direct test of DNA binding is in the Supplemental information and in my opinion would be better in the main paper.

8. The nuclease assays use three different DNA substrates but the rational for using or presenting all data is not given. One DNA substrate reports on exonuclease activity and the two others seem to report on the same endonuclease activity (hairpin or loop opening). It does not appear that any of the mutants show separation of function with the different substrates and lack or diminished nuclease activity is the only conclusion from all of the assays. Thus it does not seem useful to present all of these gels as figures. One substrate could be shown and the others put in Supplemental material to the benefit of the article as a whole.

9. The supports for the conclusion that Mre11 dimerization is needed for nuclease activity is not strong and is an already known or expected result. The differences of Mre11 in the MR complex compared to Mre11 alone need to be better explained or this also could go to supplemental information. The data shown does not strongly support the conclusions about the importance of dimerization for function.

10. In vivo assays are a nice addition but the ones shown here are a bit confusing. A better description/summary of the in vitro effects of the specific mutations tested would be useful. It is assumed that the introduced mutations effect only the specific function tested here (nuclease activity, DNA binding or dimerization). However the real possibility that in vivo these mutations result in improper MR complex assembly and thus possibly lack MR all together was not mentioned. Without some additional support for the presence of the complete MR complex in vivo when mutations are introduced, the conclusions need to be more cautions.

11. It is stated in several places in the introduction and discussion that mammalian Mre11 is involved in NHEJ pathway and some activities tested here would be functional there. There is very little effect on NHEJ in mammalian cells completely lacking MR and it is only implied to function is a specific sub-pathway where some but limited DNA end processing in needed. The Stracker and Petrini review cited does not, as implied, support MR function in NHEJ. This general ideal should be corrected. As the biological function and exact homolog relationship of the archaeal MR studied here is not known (it may be more closely related in function to bacterial SbcCD) such claims that the structure determined here is relevant for understanding function of mammalian proteins need to be carefully reviewed and cautiously written.

12. The manuscript needs a careful rewrite to correct and improve the language and clarity. The instance of missing or misplaced "the" and similar grammatical mistakes are too numerous to list here.

13. Placing this work in a larger context would benefit from discussion of a recent Paull and Tainer labs structural study, possibly published in EMBO after this article was submitted. Additionally I suggest comparing the results obtained here with a related study of Mre11 mutants that effect dimerization, MR complex stability and or nuclease activity (Limbo et al, NAR 2012).

1st Revision - authors' response 29 June 2014

Response to first reviewer's comments

1. The authors inconsistently use confusing terminology in the opening paragraphs of the results without clearly defining them. "The B-form DNA, subunit A and B, strand A and B, B-type DNA" etc. ..Maybe consider using some other abbreviations ...

>> Based on the reviewer's suggestion, we changed the terminology throughout the revised text; **(i) we changed the B-type DNA to B-form DNA; (ii) Subunit A and B → Mre11 A and Mre11 B; strand a and strand b → template and non-template strand. Also please see the Figure 1A, 1C, 1D**.

2. In general the paper is very confusing to follow given the number of mutations and the differences in residue number between organisms. Providing a diagram/summary of the location of the mutations on the subunit, the relative class of mutation (dimer interface, DNA contacts, capping domain, nuclease etc) and its equivalent place in the organisms used would be very beneficial...

>> Please see **the revised figure 3A, B, where we have combined a simplified version of sequence alignment with the cartoon representing mutations generated in this study.** We also clarified the residue number between species. Also, please Supplementary Figure 3E and 3F for the structural superposition of Mre11 proteins from different species.

3. In figure 3A, it would be helpful to point out the region and residues described in the text more clearly. Similarly, the mutations made in 3E-G could be more clearly indicated in the structure. This would be more relevant to me than the 2 figure panels dedicated to the mononucleotide binding.

>> Please **see p6 (last paragraph) and p7. We have extensively revised the description of protein – DNA in the text to improve the clarity**. In the revised text, we first described the middle region, then we described the both ends of the strand and basic region of Mre11. We also included Fig 2B for clarity of Mre11-DNA interaction. For mutational analysis, we added a new figure (Figure 3B) that contains all the information of the mutated residues. Please see also Supplementary Figure 3B and 3C for the schematic drawing for Mre11-DNA interaction. We also removed the two figure panels dedicated to the mononucleotide binding (please see below #4).

4. The relevance of the mononucleotide binding was unclear to me. Is this artifactual from the crystallization process or is there something more important that can be concluded? The argument is made that these longer DNAs are more physiologically relevant but in the context of a chromosome, I am not sure if these contacts would be relevant or whether they are simply specific to the DNA structures and process used.

>> We fully agree with a reviewer and have **removed this section from the results.**

Because we do not know (i) the exact nature of this (potential) nucleotide as pointed by reviewers, and (ii) the relevance of the nucleotide binding to whole text is somewhat unclear. However, we thought it is important to show the original electron density map for this potential nucleotide, and thus, we have included that in Supplementary Figure 1D. Also please see legend for Supplementary Figure 1D.

5. In figure 3E-G, figure 4 E-J and figure 5 A-C the authors compare mutated forms of Mre11 for activity on a number of synthetic substrates. Although conclusions are made in the text regarding relative activity, no quantification of the expected bands is provided to allow comparisons between experiments. It is also concluded that exo and endo activities are affected but how one can discern that from the gels (which products are indicative of either activity) is not immediately clear.

>> Please see **revised figures 3D, 4D, Supplementary Figure 5.** In all revised figures, we now included quantified results of the activity assay. We also have re-performed all nuclease assays with highly purified substrates and proteins. Based on the suggestion by third reviewer, we removed the assay with the substrate HP2 (for redundancy) and moved the exonuclease assay result to supplementary Figure 5. In this way, endo (DAR134) and exo (TP580/124) products should be clear.

6. In the same figures, no evidence that the proteins are added in equivalent amounts is presented.

>> We have included **a gel at the bottom of each assay result.** This shows the equivalent amounts (or equally scaled amounts) of proteins are used in the reaction.

7. In figure 4, it would be helpful to the reader if the structural features described, namely the capping domain and four helix bundle, were clearly indicated.

>> In the revised figure, **we have indicated the capping domain and four-helix bundle. Also by showing the close-up view in the same orientation (Fig 4B)**, we improved the clarity.

8. In figure 4, is there evidence that these mutations affect the rotation of the capping domain or is this assumed based on the structure?

>> Please see **revised paragraph (P8 last paragraph, P9 first paragraph, p10 line 6 to 8).** Rigid-body rotation is from the structural observation. The reduced and oxidized crosslinking mutation affects the dimer stability and possibly the quaternary structure. We clarified this part and more precisely described.

9. In figure 5, it is unclear why the experiments are now done in the context of Rad50

rather than with Mre11 alone as in all previous experiments. It makes it somewhat difficult to compare.

>> We have used the MR complex mainly because the **solubility** of this Mre11 alone mutant is very low (presumably because of the dimer to monomer perturbation) and we can only obtain the small amount of mutant protein for assay even in the presence of Rad50. However, **based on the suggestion by third reviewer 3 (Q9), we removed this part.**

10. A major issue to consider in the interpretation of the yeast data is the status of the MRX complex. Previous conclusions regarding nuclease mutations were later recanted as the mutations disrupted the whole complex creating essentially a null situation. The authors should provide evidence that the interactions with Rad50/Xrs2 are not disrupted as the interpretation of the data hinges on knowing this information.

>> Please see the **revised section (p12, line 3 – 9 (or second paragraph) and figure 6A and 6B).** We have used both co-immunoprecipitation and two-hybrid assays to demonstrate that both K62A ad R184A mutations fully support the assembly of MRN *in vivo*. The observed NHEJ and resection deficiency of mutant mre11 expressing cells is not unlikely due to unstable complex formation. Due to the inability to fully confirm the integrity of complex assembly expressing mre11-K83A, we have removed the part describing the mre11-K83 in our revised manuscript.

Response to second reviewer's comments

Abstract:
1) Please mention the source organism, M. jannaschii.

>> Please see **P2, line 7. We have corrected the abstract accordingly.**

2) '....using longer DNA molecules that more accurately reflect damaged DNA,..'. Perhaps change to "....using longer DNA molecules that more accurately reflect a broken chromosome"
>> We truly thank to a reviewer for this comment. We **changed the sentence** as a reviewer has suggested..

Introduction:
3) I suggest to rename the Mre11 complex to Mre11-Rad50 complex.

>> Please see P3, line 2. We **renamed to the Mre11-Rad50-Nbs1 complex** (or the MRN complex) as suggested by a reviewer

4) The three different models (and figure 1) should be left out or their origin needs to be clarified.
>> We have **removed the figure 1** and its description in the text.

Results:
Overall structure of the DNA bound MjMre11 complex:
5) The DNA substrates used for crystallization could be named DNA1 and DNA2 to make things clearer.
>> Please see Figure 1A and P5 (line 6, 7). We renamed the DNA accordingly

6) Supplementary Table 1:
The overall statistics are fine, but there are some issues and some missing details:
- The units for r.m.s. deviation of bond angles should be degrees ({degree sign}), not Å.

>> **Please see a revised S. Table 1.** We have corrected it.

- It appears that the Ramachandran plot was analysed using Procheck. As Procheck is outdated, it would be preferable to use Molprobity. It would also be recommendable to reference the program used for validation (Procheck, Molprobity or anything else).

>> We have recalculated the Ramachandran plot using Molprobity

- The table footnotes define the r.m.s. deviation in B factors between bonded atoms. However, there seems to be no such entry in the table itself. Please amend.
- Please also list I/σ.

>> **We have included I/sigma.**

- Given the low resolution of the structure, it would be helpful to list the resolution at which I/σ {less than or equal to} 2 and Rsym {greater than or equal to} 50 %.

>> **We have included Rsym (< 0.5) in the table**. The table contains resolution where I/sigma is near 2.

- Please state average B-factors for protein, nucleotide and DNA atoms.

>> **We have included averaged B-factors** for protein, DNA, Mg atoms.

- Please correct typographical errors such as "relections", "generouslly" etc.

>> **We have corrected them all**.

7) The authors do not allow the reader to make any judgment of the quality of the structure. Given the low resolution, it would be helpful to provide examples of electron density (omit maps, 2Fo-Fc maps) for important parts of the Mre11 protein (e.g. the Mg site and DNA interacting loops), for bound DNA and for the bound nucleotides.

>> Please see the **revised Supplementary figure 1A-D**. We have included the four maps for the DNA, Mg site, protein-DNA interface, and the bound (putative) nucleotide.

8) The authors claim there are almost no differences between the MDB1 and MDB2 co-structures, "except a few additional DNA contacts with Mre11 in Mre11-MB2". This phrase is intriguing.
- What are these differences? How could they come about? Could they tell us something about different binding states?

>> Please see **Supplementary Figure 3A, 3B, 3C. We have included the structural comparison between DNA1 and DNA2**, and interactions between Mre11-DNA1 and –DNA2. **Please see P6 (second paragraph).**

- Could the authors present an superposition of these two structures

>> Please see **Supplementary Figure 3A.** We have included a superimposed structure of Mre11-DNA1 and Mre11-DNA2

- In that context, only one Mre11 dimer out of three in the a.u. seems to have bound DNA. Is it possible that the DNA molecule mediates crystal contacts?

>> DNA does **not make any contact with symmetrically related DNA or Mre11.** We described it in P5 (line -9) or end of a second paragraph.

- In the latter case, can the authors exclude that the differences in DNA contacts are a function of slightly different crystal packing?
>> In both Mre11-DNA1 and Mre11-DNA2, **crystal packing is same**.

9) Sung et al. compare their structure only to the so-called synaptic DNA-Mre11 complex published by Williams et al 2008. However, Williams et al. also reported a second DNA-Mre11 complex, a branched DNA complex, which the authors chose to ignore completely. How does the DNA complex solved by Sung et al. compare to this second complex? In that context, the authors should rethink the three models they postulate in the introduction and may have to rewrite their discussion and structural comparisons.

>> Please **see p4 (line 4-13 or first paragraph). Figures 2A, 2B, 2D, 2E, p12 (line -6).** We have revised introduction and discussion.

10) Materials and methods:
- Protein Expression and Purification: The paragraph lacks important information, such as primer sequences used for cloning, expression conditions, cell disruption, buffer compositions and purification (type of columns etc.). Please correct.

>> Please see the **revised introduction (Protein Expression and Purification),** where we have included all related information

- Structure determination and refinement: The authors state that both crystal forms contain one dsDNA-MjMre11 complex in the a.u. However, according to the results part, an a.u. contains three Mre11 dimers and one DNA. Please correct.

>> Please see **P16, (third paragraph & line 1).** We have corrected the description.

- During refinement, NCS restrains were applied. Was NCS strictly enforced, even in the case of the DNA-bound Mre11 dimer?

>> Please see **P16 third paragraph (or last line). We have applied restrained (not strict) NCS throughout the refinement process**. We thought that the considering the resolution of the data, applying NCS is the best way to increase the reflection/ parameter ratio during refinement process. We also tried to release the NSC restraints at the last stage of refinement. However, both refinement statistics and geometry did not improve.

11) Figure 2A and B
- The imposition of DNA from the PfMre11-DNA structure should have been presented in a separate figure to avoid confusion.

>> Please see **the revised Fig 1E and 1F**.

12) Figure 3 A-D
- Most of the structural figures are inconsistent when it comes to color schemes and different viewing angles. Further, close-up figures and better labelling (active site etc.) would also be helpful for the general readers.

>> In the revised **Fig 2 (and Fig 1) and all others, we tried to make the figure in consistent color.** We also tried to fit the angle as close as we can, but in some case, for clarity we had to change the angle.

DNA recognition by Mre11:
13) Supplementary Figure 3:
The Mre11 sequence alignment needs to be corrected:
- The color scheme seems to be misleading. Yellow is supposed to indicate "strictly conserved residues", according to the figure legend. ..Please redo the labelling of similar and strictly conserved residues in your alignment, perhaps using programs such as Clustal Omega and Jalview.

>> We thank a reviewer for this important comment. We realigned using **Clustal Omega. We have included a simplified alignment in the figure 3A** (as suggested by the first reviewer Q2), and removed the whole alignment from Supplemental Figure 3.

- It is surprising that, in the case of human Mre11 (hMre11), ... Please use the native hMre11 sequence as available on Uniprot or GenBank.

>> Please **see a legend for Fig. 3A.** We have included all the information as suggested by a reviewer

- In case of SpMre11, it seems unlikely that the first residue is an A. Please adjust the sequence numbering (or the sequence).
- According to the legend, nuclease motifs are highlighted using brown. It may be a printer/screen problem, but these boxes appear rather to be red.

>> The color scheme has been **changed and the boxes are yellow and red**

- In the legend, please define the sequences, referencing the source organism and a UniProt or NCBI (or similar) accession number (e.g "MjMre11, M. jannaschii, UniProt accession number XXXXXX").

>> We have included the **suggested information in the Figure 3A legend**.

14) Page 6, last paragraph:
- The authors mention that residues such as Y13, R55 and R89 can be imposed on PfMre11 structures and refer to Fig 3A and SI Fig3. However, these figures do not show a structural alignment although such a structural superimposition would be useful and should be added by the authors (also for MjMre11 vs hMRe11 and vs yeast Mre11).

>> Please see revised **supplemental figure 3E and F.** We have included the superimposed figure. In these figures, we have superimposed all reported structures of Mre11.

Additional interactions between MjMre11 and Nucleotides:
15) Page 6:
- 'We also observed three mononucleotides bound to an MjMre11 subunit other than the DNA-binding units'. The word "subunit" does not seem to be the right term and is actually confusing. The authors seem to refer to the three Mre11 dimers in the asymmetric unit, and they should put it this way.
- The authors should show the binding sites of these nucleotides in an overview figure to make it easier to

>> **We have removed this section for the following reasons; because we do not know (i) the exact nature of this nucleotide as pointed by reviewers (specially the first and second reviewers'), and (ii) the relevance of the nucleotide binding to whole text is somewhat unclear**. However, we thought it is important to show the original electron density for this potential nucleotide, and thus, we have included that in **Supplementary Figure 1D.** Also please see legend for Supplementary Figure 1D.

16) An interesting finding is that nucleotides are bound by non-DNA-binding Mre11 dimers. The authors postulate that these nucleotides may be derived from the co-crystallized DNA. However, the authors should consider following scenarios and should test them:
- The authors may well be right and the nucleotides are derived from the DNA ligands. The authors could test this hypothesis by analyzing the DNA before and after incubation with MjMre11 and Mg2+.
- The nucleotides may be contaminants introduced when adding DNA for co-crystallization. Did the authors purify their DNA samples, e.g. using HPLC or PAGE?

>> Please see **the revised "DNA oligonucleotide and 32P labeling" section** in Supplementary Materials and Methods section. The DNA used for crystallization was highly purified (twice) by PAGE. Nevertheless, as **a reviewer pointed out, we do not know the nature of the electron density and approached more conserved way** to describe this part in the revised manuscript.

- The nucleotides may have been co-purified from E. coli. In any case, the authors may be able to identify the nature of these nucleotides using methods such as mass spectrometry or

NMR. Unfortunately, the authors don't discuss these nucleotides any further. They, in contrast to the bound DNA ligands, may yield clues about the biochemical mechanism of Mre11, as one of them is actually close to the active site.

>> **Even if we identify a nucleotide by mass spec, it does not mean the particular nucleotide is the one for this density (specially at this resolution),** and we conclude that it is better not to describe the model in detail and just to provide electron density map for the corresponding region. Furthermore, we already know the active site metal and geometry (with the help of Hopfner et al (2001) structure) and thus, we conclude that adding a nucleotide does not add new clue for the mechanism.

Mutational analyses:
17) The authors claim that Arg89 is a conserved residue involved in DNA-binding. If compared to the multiple sequence alignment (Suppl. figure 3), Arg89 is absolutely not conserved (nor similar) to the other Mre11 homologs, which harbor either a Q, S o T residue in the same position. Intriguingly, the authors generated an R89S mutant, which showed no more nuclease activity. The authors gloss over the fact, however, that SpMre11 and ScMre11 retain their nuclease activity despite having an S in exactly the same position. Please discuss.

>> Please **see P13, second paragraph. We have fully discussed this point.** After several additional times of repeated assays, the R89S mutant contains some activity rather than null (although significantly reduced activity). See the revised figures (Figure 3D, Supplementary Fig 5). First, the activity we measured is **relative activity** to the wild type Mre11 and the weak activity does not mean that the mutant does not have activity. Second, the **corresponding residue is disordered** and slightly different position in **eukaryotic Mre11**. Third, it is possible that the interaction between eukaryotic Mre11 – DNA may not the same as those of archaeal Mre11 – DNA (Supplementary Figure 3E).

18) Materials and methods:
- A 'DNA oligonucleotides and P32 labelling' chapter should be included, containing information about DNA sequences, labelling - and annealing procedure. The DAR134 substrate seems to contain two different hairpin conformations (double bands), and hence an additional purification step is required. The purification can easily be performed by separating the annealed substrates on a 10 % native PAGE, followed by gel excising and elution (diffusion) into H20. Also, could the samples have been separated on a 20% sequencing gel to obtain a better resolution? Information regarding the detection method is also missing.

>> Please see the **revised Supplementary Materials and Methods (and figures 3D, 4D, and Supplementary Figure 5).** We have included a “'DNA oligonucleotides and P32 labelling” section. We also have re-prepared (including additional purification step as suggested by a reviewer) all the samples (two substrates DAR134 and TP124/TP580, as well as proteins) and re-performed all the assays and separated samples using a 20% urea

gel. We note that the exonuclease assay is included in Supplementary Figure 5 as suggested by the third reviewer and we removed the assay using the HP2 substrate as it is redundant with other substrate.

- The sizes of the nucleolytic degradation products are labeled in all figures, however, the size marker itself is not shown. Please include the size marker, at least in form of a supplementary figure.

>> **Please see Figures 3D, 4D, Supplementary Fig 5.** We have included size marker.

- The authors have not included any information about sequences of the quick change primer nor the site-directed mutagenesis protocol. Please provide this information.

>> Please see Supplementary Table 4 for the primer sequences and “mutagenesis” section in Supplementary Materials and Methods.

19) Figure 3 E-G

- A small part of the gel (above the substrate bands) should be shown, to get an idea of the purity of the various DNA substrates. This is true for all the nuclease assay gels.

>> **Please see the Figure 3D, 4D, and Supplementary Figure 5**. We included whole gel.

- It would also be informative to include quantitative analysis of the substrate degradation using ImageQuant etc. for all nuclease assays.

>> Please **see the Figure 3D, 4D, and Supplementary Figure 5.** We have included all the quantified results on right of each figure.

- Fig 3F: As mentioned earlier, the DAR134 substrate needs to be purified to avoid the two different hairpin conformations.

>> We have **purified DAR134 twice to avoid different hairpin** conformations.

- Fig. 3G: The figure has a very low resolution and should be optimized in order to draw any conclusions. It seems that less substrate could have been added in the rxn's containing K129A and K132D, and if so the activity of the Lys mutants would be more similar to wt.

>> We **have increased the resolution of the gel as much as we can**. Please see the bottom of each figures where we have included the amount of protein on the SDS-PAGE

gel.

20) Supplementary fig. 5A
- The band shifts are stacked in the wells and have not migrated into the gel. Why haven't the P32-labeled substrates been used and detected by phosphor imaging, as for the nuclease assays? Could the complexes have been separated on a lower percentage native PAGE or an agarose gel in order to avoid the well shifts? Also, DNA binding could have been verified by Biacore or filter binding assays to detect binding affinities of dynamic DNA- protein complexes.

>> Please see **the revised Figure 3C. We have used both P32 labeled DNA and 6% native PAGE** and resolved the problems raised by a reviewer.

21) Lys132 is a recurring theme in this paper, partly because it "is close to DNA" and partly because it is deemed a "conserved basic residue". According to the alignment in suppl. Figure 3, this residue is a tryptophan in PfMre11, which seems to contradict somewhat the notion of conserveness. Also, the authors should make their case for a role in DNA processing more plausible. What is the exact orientation of Lys132 towards DNA? What kind of interaction do they expect? All that information cannot be derived from the figures.

> Please see **Supplementary Figures 3F and 4. Although the Lys132 and other residues are not conserved in sequence, they are structurally conserved.** We clearly described this information in P7 (second paragraph). Also please see Figure 2A, 2B, Supplementary Figures 3F. They are toward the phosphate ion but distance is 3.9 A. We have **described this in the text (P7 (second paragraph)).**

Quaternary structural changes of Mre11:
22) Page 8, 2nd paragraph.
- 'To examine conformational changes of Mre11, we compared the structures of five MjMre11 dimers (three dimers in this study; one DNA-free MjMre11, PDB 3AUZ; one MjMre11 bound to Rad50, PDB 3AV0; Lim et al, 2011).' The paragraph is somewhat confusing. Is only the alignment of MjMre11-DNA towards DNA-free MjMre11 (PDB 3AUZ) shown in figure 4A, or is the MjMre11-Rad50 (PDB 3AVO) also included in the structural comparison? Information about the different structures (and PDB codes) should be included in the figure legend.

>> Please **see P9 (first paragraph, line 6) and Figure legend (Figure 4A).** We have clarified the sentence by including additional information (Mre11 A/B vs Mre11 E/F). We have included Mre11 A/B and Mre11 E/F in the figure for clarity and described it in the legend.

Cross-linking of a dimeric interface increases the nuclease activity:
23) Figure 4:
- It is not clear which samples were under reducing or non-reducing conditions. Please

add a label or a description mention in the figure legend.

>> The **figure (4C and Supplementary Fig 5B) contains DTT (reducing agent) and H2O2 (oxidizing agent) on top.**

- Fig. 4F: As for figure 3F, the DAR134 substrate needs to be purified prior to nuclease activity measurements.

>> We have **purified DAR134 and redone all the assays** for several times.

- Fig. 4G: As for figure 3G, the assay has too low resolution and the amount of substrate added to each reaction does not seem to be consistent.

>> We have **included exactly same amount of substrate and proteins**.

- Fig. 4I: Even though the inactivity of the I302Y mutant is obvious (at least when comparing the amount of substrate), the DNA substrate (control) is degraded or contaminated, and consequently the experiments should be repeated using a purified substrate.

>> Please see **the Figure 3D (Supplementary Figure 5)**. We have repeated the assay at least three times using a purified substrate.

24) Page 9, 1st paragraph:
- 'Importantly, this mutant showed comparable or ..It should be mentioned that di-sulphide bonds are formed during oxidizing conditions (and consequently a stable Mre11 dimer), to make it easier for the reader to understand the conclusion.

>> Please **see p10 (line 2).** We have changed the sentence accordingly. We note that additional information is also included in above paragraph

Mre11 Dimerization is critical for nuclease activity:
25) Figure 5:
- Please provide information about protein concentrations, as well as labels of the different DNA substrates.

>> We **have removed this section based on the comment by the third reviewer (Q9).** We can include this section upon reviewers' suggestion. However, this section does not seem to add any new information based on the third reviewer's comments and we removed it.

- The authors do not provide any details about the protein expression and purification of MjRad50.

>> Please see the comment above.

- All the figures have a very low resolution, and thus the authors should be cautious drawing too many conclusions. Eg: For the DAR134 substrate it is clear that the dimeric mutant actually has a nuclease activity, at least when compared to the control sample. However, the nucleolytic degradation pattern differs when comparing the dimeric mutant to wt.
- 'Although it is not completely abrogated, the "dimeric" mutant showed significantly decreased exonuclease activity compared to wild type MjMR toward TP124/580 in the absence and presence of ATP (or ATPγS) (Figure 5A, lane 4-7).' The only significant reduction in endonuclease activity can be seen for APO (Lanes 2 and 3), and not for ATP/ATprS as stated by the authors.

>>Please see above comment. **We have removed this section because this ection does not add much information based on a reviewer' comments**. Also, in our original text, we clearly wrote that dimeric mutant did cleave substrate (reviewer pointed out the mutant has activity as we described). We note that in the presence of ATP, MR showed reduced nuclease activity even in the wild-type as it blocks the active site.

- Fig. 5C: The resolution of the image is far too low, and the result should be optimized prior to publication.
>> Please see the above comments..

In vivo analysis:
26) It should be clear which yeast strains (genetic background) have been used in the different in vivo assays.

>> We have modified the **figure legend (Supplementary Fig 5-8) and Supplementary Materials and Method, Supplementary Table 3** to clarify the yeast strains used in each experiment.

27) Figure 6 B: please correct the labeling of the axis to the correct percentage or does the wildtype has only 0.3 percent survival in Figure 6B ?

>> Imprecise NHEJ is very inefficient in budding yeast. Even in Mre11+ cells, only 0.3% repairs HO induced break by imprecise NHEJ. **0.3 percent survival is well within the expected value by this assay in wild type yeast (please see Moore and Haber, 1996 MCB 16:2164 and Lee et al, 2002 DNA repair 1:27-40)** and the mutation in *mre11* (K62A and R184A) reproducibly decreased NHEJ frequency to 1/3 or 1/2 of the wild type yeast in this assay.

28) Figure 6D
- The spot assays on the lower part of the figure must have been incubated for a shorter period of time if compared to the spot assays in the upper panel. This is easily observed

when comparing the phenotypes of sgs1Δ (1) with sgs1Δ (6). Thus, the authors are strongly encouraged to repeat the spot assays, in which all the stains should be spotted on the same plates and tested towards both CPT and phleomycin. There is no need to spot the same strains twice as seen for both nr (1) and (6), and (5) and (7), respectively. Further, the number of parallels should also be mentioned in the material and methods.
- It is not clear whether mre11 sgs1Δ (2) is an mre11 deletion mutant or not. Please label properly.

>> **Please see revised Fig 5D and Supplementary Figure 6.** We redid all the spot assays and replaced the previous versions with the new ones that were subjected to equal amount of culture in 30^{o}C (3-4 days). We have also modified the Figure legends to indicate the number of parallels performed in spot assays. We have modified the label to *mre11*Δ *sgs1*Δ to accurately indicate it is an *mre11* deletion.

29) Figure 6 E-F: Please comment
>> **Please see P11 (line -5 to -3; Fig 5E - 5G).**

30) Page 10, 3rd paragraph:
- "....., we deleted SGS1, the gene required for one of the two.....". Please be more specific, both when it comes to the protein the gene codes for and the resection pathway.
>> Please see **P11, (second paragraph & line 3-5).** We have modified the manuscript to accurately describe genes and the proteins they code for.

Discussion:
31) The authors claim that they disrupted the MjMre11 dimer interface by introducing "bulky hydrophobic residues". However, the triple mutant they used introduces only one bulky hydrophobic residue, but one alanine and one arginine. Please correct.

>> We have **deleted this sentence as we removed the corresponding** data.

Response to the third reviewer's comments

1. It would be interesting to compare the DNA-protein interface identified here with other DNA-protein interfaces, particularly others where the DNA is similarly distorted.

   >> Please see **P7 (last paragraph), P8 (first paragraph),** and Figure 2F. We have compared with PfMre11-dsDNA, which exhibited similar distortion of a minor groove (expansion) and p53-DNA, in which the minor groove of DNA is rather compressed.

2. It would be interesting to report more information and better describe the three different dimer forms in the asymmetric unit. .. The structural description part of the paper would be stronger with these elements expanded. In general a clearer coherent description of the structural elements revealed here would help.

>> Please **see P5, (second paragraph) and Supplementary Figure 2A and 2B, as well as Figure 1B**. We have fully described the protein-protein interaction.

3. The description of the DNA fragment bound across Mre11 is very confusing. Referring to right and left halves of the DNA is not helpful or even accurate. I do not know what this means. . DNA as "a" and "b" and to the two Mre11 protein subunits of the dimer are "A" and "B" is not at all a good choice. It would be useful to reconsider the wording and describe .. compared to the previous structure which included short oligos that could not bind across the whole dimer.

>> Please see **p5, line 8; P6 (last paragraph) and P7.** We have rewritten the whole section with more clear terminology. (i) we changed the B-type DNA to B-form DNA; (ii) Subunit A and B → Mre11 A and Mre11 B; strand a and strand b → template and non-template strand. Also please see the Figure 1A, 1C, 1D.

4. Reference to base positions with 3 letter abbreviations, p. 5 Cyt, Thy, Gua, is non-standard and confusing. Similar structural work usually refers to bases with one letter and a number indicating position along the DNA strand sometimes with or without a ' to indicate different strands.

   >> Please see the **revised fig 1A. 2A, 2B**. We have redefined the number of bases in DNA.

5. The structural bits shown in some figures are hard to understand. For instance in figures 3 B,C and D, and 4 B and D, it is very difficult to figure out where these parts of the structure fit into the whole. Better figures and description are needed.

>> Please see **the revised Figure 4B**. We have used same orientation as 4A. We also changed **Fig 2C (formally 3B) to the same orientation as Fig 2A**. We have removed

Figure 3 C and D based on the first reviewer's concern. Because we do not know (i) the exact nature of this nucleotide as pointed by reviewers, and (ii) the relevance of the nucleotide binding to whole text is somewhat unclear. However, we thought it is important to show the original electron density for this potential nucleotide, and thus, we have included that in **Supplementary Figure 1D**. Also please see legend for Supplementary Figure 1D.

6. Figure 1 is not necessary and not informative. Where as figure S1 is in my opinion much more useful information and would be appreciated in the main paper.

>> We have **removed Figure 1 and moved Fig S1 to Fig 1 A and 1B.**

7. The mutations made in the newly discovered DNA binding site are tested for DNA binding by an activity assay. Presumably if DNA is not bound then it cannot be acted upon by the protein it does not bind to. The direct test of DNA binding is in the Supplemental information and in my opinion would be better in the main paper.

>> Please **see Fig 3C**. We have re-performed the DNA binding test using a radioisotope labeled substrate using 6% native PAGE gel.

8. The nuclease assays use three different DNA substrates but the rational for using or presenting all data is not given. One DNA substrate reports on exonuclease activity and the two others seem to report on the same endonuclease activity (hairpin or loop opening). ... Thus it does not seem useful to present all of these gels as figures. One substrate could be shown and the others put in Supplemental material to the benefit of the article as a whole.

>> Please **see Figure 3D, 4D, 4E, and Supplementary 5.** We have included endonuclease (DAR134) in the main figure and moved exonuclease results (TP124/580) to Supp. Fig 5. We have **removed HP2 results.** We note that we **have re-performed and quantified all the assay as suggested by second reviewer**. We note that because of the space availability (or figure organization) in Figure 4, we have included both endo- and exo-nuclease activity analysis in D and E.

9. The supports for the conclusion that Mre11 dimerization is needed for nuclease activity is not strong and is an already known or expected result. The differences of Mre11 in the MR complex compared to Mre11 alone need to be better explained or this also could go to supplemental information. The data shown does not strongly support the conclusions about the importance of dimerization for function.

>> We have **removed the perturbation data (Formally Fig 5)** base on this comment.

10. In vivo assays are a nice addition but the ones shown here are a bit confusing. A better

description/summary of the in vitro effects of the specific mutations tested would be useful. It is assumed that the introduced mutations effect only the specific function tested here (nuclease activity, DNA binding or dimerization). However the real possibility that in vivo these mutations result in improper MR complex assembly and thus possibly lack MR all together was not mentioned. Without some additional support for the presence of the complete MR complex in vivo when mutations are introduced, the conclusions need to be more cautions.

>> Please **see P12 (line 3-10), second paragraph**, and Fig 6A and 6B. We have examined the assembly of the MRX *in vivo* **using co-IP and yeast two-hybrid analysis**.

11. It is stated in several places in the introduction and discussion that mammalian Mre11 is involved in NHEJ pathway and some activities tested here would be functional there. There is very little effect on NHEJ in mammalian cells completely lacking MR and it is only implied to function is a specific sub-pathway where some but limited DNA end processing in needed. The Stracker and Petrini review cited does not, as implied, support MR function in NHEJ. .. relevant for understanding function of mammalian proteins need to be carefully reviewed and cautiously written.

>> Please see P14, second paragraph. We have addressed the reviewer's concern by including a sentence "**the role of MRN in NHEH in vertebrate cells is not yet fully defined**". In addition, we have rewritten both introduction and discussion sections to fully disclose the uncertainty associated with the role of Mre11 complex in NHEJ in vertebrate cells.

12. The manuscript needs a careful rewrite to correct and improve the language and clarity. The instance of missing or misplaced "the" and similar grammatical mistakes are too numerous to list here.

>> The manuscript has been English edited by the two native speakers independently.

13. Placing this work in a larger context would benefit from discussion of a recent Paull and Tainer labs structural study, possibly published in EMBO after this article was submitted. Additionally I suggest comparing the results obtained here with a related study of Mre11 mutants that effect dimerization, MR complex stability and or nuclease activity (Limbo et al, NAR 2012).

>> We thank to the reviewer for this comment. Please see the revised introduction (**P3, third paragraph**) and results (**P12, second paragraph**), and discussion (**P13, third paragraph**). We have discussed and described the results of Mre11 of Limbo et al (NAR, 2012) and Despenda et al (2014).

1st Editorial Decision 17 July 2014

Thank you for submitting your revised manuscript on Mre11 DNA recognition for our consideration. It has now been assessed once more by the original referee 2 (see comments below), and I am pleased to inform you that s/he is satisfied with the revisions and considers the study now suitable for publication in The EMBO Journal.

Before proceeding with formal acceptance of the manuscript, I would like to ask you for several minor additional changes:

- please upload higher resolution/less compressed files for the 6 MAIN figures - maybe using TIF instead of JPG format - to ensure that the structural views can be well reproduced. Also, please upload source data blots for Fig 6A whose quality is currently too low to properly assess it. For Fig 6B (right panel), please make sure to indicate any separation between spots that may not have been directly adjacent on the original plate.

- Finally, I would propose to incorporate the following minor changes to the abstract:

The Mre11-Rad50-Nbs1 (MRN) complex plays important roles in sensing DNA damage, as well as in resecting and tethering DNA ends, and thus participates in double-strand break repair. An earlier structure of Mre11 bound to a short duplex DNA molecule suggested that each Mre11 in a dimer recognizes one DNA duplex to bridge two DNA ends at a short distance. Here, we provide an alternative DNA recognition model based on the structures of Methanococcus jannaschii Mre11 (MjMre11) bound to longer DNA molecules, which may more accurately reflect a broken chromosome. An extended stretch of B-form DNA asymmetrically runs across the whole dimer, with each end of this DNA molecule being recognized by an individual Mre11 monomer. DNA binding induces rigid-body rotation of the Mre11 dimer, which could facilitate melting of the DNA end and its juxtaposition to an active site of Mre11. The identified Mre11 interface binding DNA duplex ends is structurally conserved and shown to functionally contributes to efficient resection, non-homologous end joining and tolerance to DNA damaging agents when other resection enzymes are absent. Together, the structural, biochemical, and genetic findings presented here offer new insight into how Mre11 recognizes damaged DNA and facilitates DNA repair.

After these final modifications, we should be able to swiftly proceed with formal acceptance and production of the manuscript. Thank you again for this contribution to The EMBO Journal.

Referee #2:

The authors made a substantial effort to address my comments. All in all, the manuscript is very much improved. I can recommend publication of this important contribution to our understanding of the Mre11 protein.

2nd Revision - authors' response 20 July 2014

Thank you very much for your encouraging comments and decision. We are very happy about your decision. We are enclosing the revised manuscript along with main-figures in high resolution and a sheet for highlights.
In the revised manuscript, we have changed the abstract as you have suggested. In addition, we included main-figures in high resolution tiff formatted files. We have included source files for Fig 6A and Fig 6B. For 6B, the spots are all from the original plates without any modification - there are weak background spot (in the right panel). If you suggest any modification, we are happy to follow your suggestions.

