

Structure guided functional analysis of the *S. cerevisiae* Mre11 complex

Corresponding Author: Dr John Petrini

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The Mre11 complex (containing the Mre11-Rad50-Xrs2 subunits) has important functions to promote DNA break repair and activate the DNA damage response signaling cascade dependent on Tel1. We now know quite a lot about the function of the Mre11 complex from genetic and biochemical studies. However, the molecular details, particularly how the structure of the Mre11 complex drives its function, remain elusive. The authors' achievement to obtain a high-resolution structure of the yeast Mre11-Rad50 is therefore important. My enthusiasm about this work is however reduced by a lack of functional insights and by the sometimes confusing mutant analysis.

Specific comments

- Structural information on the Mre11-Rad50 complex is to date largely based on data from bacteria or archaea. Work from several laboratories (and particularly KP Hopfner) indicated that the Mre11-Rad50 complex assumes various conformations. The authors' structure presented here appears to be a combination of the "active" and "inactive" forms with respect to Ref10. The coiled coils are clamped together (as in the active form), yet Mre11 remains as the base of the structure (as in the inactive form). It is not clear whether the ATPase-dead mutation in Rad50 used here impacted the structure. I understand the author's motivation to reduce sample complexity, yet, as a result, it is not clear whether the obtained structure is a physiologically relevant intermediate.
- The authors identified and described the interface between Mre11 and Rad50, and designed a number of mutant combinations to validate the structure. Some of the mutations however disrupted Mre11 homodimerization, as well as interaction with Xrs2 (bottom part of Fig. 2). I am puzzled by this analysis: the mutations at this point do not validate the structure, what do they serve for? Mutations disrupting interactions with Xrs2 may disrupt nuclear import of Mre11-Rad50 and further complicate interpretation of CPT sensitivity experiments. The rationale of the experiments is not always clear.
- We learn very little about the function of the Mre11 complex. For example: Figure 3: It is well known that homodimerization of Rad50 occurs also through the ATPase domain. Figure 4: The authors observed multiple contacts between Rad50 and dsDNA. Mutating these residues individually had little impact on the protein function as scored by CTP sensitivity or DNA binding. Only combination of mutants resulted in defects. However, some of these conserved residues have already been implicated in dsDNA binding in various organisms (particularly by the Hopfner lab). Figure 5: It is also well known that the ATPase activity of Rad50 is necessary for the removal of Spo11 and meiotic progression. Although the identified 3KA mutant was not described before, the impact of disrupted ATPase is known. The manuscript would profit from shortening and focus on novel insights.

Provide reference for: "It is well established that defects in dsDNA binding by Rad50 confer sensitivity to clastogenic insult."

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In this study, the authors report a 3.2 angstrom resolution cryo-EM structure of the *Saccharomyces cerevisiae* Mre11-Rad50

complex with bound double-stranded DNA (dsDNA). Based on the structure, residues at protein interfaces (homo and heterotypic), and of Rad50 in contact with dsDNA were systematically mutated and tested for DNA damage resistance, complex formation, DNA binding and ATPase activity. The authors identified a single Rad50 residue that is critical for function at the interface with the Mre11 HLH domain, whereas multiple mutations at the interface with the Mre11 capping domain conferred only mild DNA damage sensitivity. Interestingly, they found that the interface between the Mre11 capping domain and Rad50 is important for assembly/stability of the MRX complex. Not too surprisingly, mutation of single residues that contact dsDNA did not confer a phenotype or only mild CTP sensitivity, but combinations of these mutations reduced DNA binding and DNA damage resistance. The authors also identify three lysine residues of Rad50 that are near the dsDNA binding surface and are critical for Mre11 nuclease and ATPase activities. The authors suggest that these residues could be important for transition from the closed to open complex. Analysis of suppressors of the rad50-3KA mutant support the idea that the Rad50 coiled coil domains influence the enzymatic activities of the globular Rad50 head domain.

This is a comprehensive study that provides new insight into the role of conserved residues of Mre11-Rad50 that are critical for function and are frequently mutated in cancers.

Comments:

1. Was Xrs2 interaction with Mre11 tested in the rad50-D1126A mutant?
2. Line 219, "Rad50-D1126 of the HLH domain"? Isn't the HLH domain in Mre11?
3. It would be important to measure the ATPase activity of the Rad50-P168S single mutant to see if it is higher than WT. It is possible that this mutation increases ATPase activity independently of the -3KA and -46 mutations.
4. The E356K mutation is the best suppressor of rad50-3KA CPT sensitivity. Was this mutation tested for suppression of the rad50-3KA ATPase activity? Also, does it suppress the rad50-46 phenotype?
5. Line 405. Are authors suggesting that the threshold of ATPase activity is lower for Mre11 nuclease activation than for Tel1 activation?

Reviewer #3

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Hohl and colleagues present a 3.2 Å cryo-EM structure of the *S. cerevisiae* Mre11-Rad50 complex with bound dsDNA, representing a closed form, in addition to mutagenesis and functional analysis. They reveal new features of the Rad50-dsDNA interaction interface, and inter- intra protein contacts important for assembly and dynamics.

The quality of the data appears OK, and I have no major issues with the analysis. The authors recognise limitations to the study - which is a shame they were unable to obtain MRX particles and WT MR, but I still believe the data they present to be significant.

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Figure 1B – Might be easier to view if the two protomer of Rad50 and MRE11 are slightly different shades of blue and pink (to match to the domain scheme in Figure 1A) rather than using completely different colours for the protomers, it is a little confusing.

Why was 83-mer double stranded DNA (dsDNA) chosen?

Page 6- Last paragraph. Could the authors please clarify/expand when they mention they were unable to obtain useable MRX particles? Do they believe they were too heterogenous, flexible or aggregated? Also what is meant by "confounding effects" of Xrs2?

Version 1:

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My suggestions were aimed to improve the flow of the text, focus on novel insights, and I thought that the description of mutant analysis was not very clear.

For example, mutations in the Mre11 or the Rad50 subunits may affect the function of these proteins per se, or may disrupt interactions with Xrs2. In the latter case, lack of interactions with Xrs2 would lead to a disruption of nuclear import of the Mre11 and Rad50 subunits (NLS is located on Xrs2). Both of these scenarios could lead to CPT sensitivity. Hence, I was asking the authors to clarify this point, and the authors replied that there is "no ambiguity". A more specific reply and clarification would be helpful. To fully distinguish between the two scenarios, one would have to e.g. place NLS on MRE11,

as was done in the Symington lab. I would be satisfied with a cleared description of potential limitations.

With respect to the novelty, I raised the following points: "We learn very little about the function of the Mre11 complex. For example: Figure 3: It is well known that homodimerization of Rad50 occurs also through the ATPase domain. Figure 4: The authors observed multiple contacts between Rad50 and dsDNA. Mutating these residues individually had little impact on the protein function as scored by CTP sensitivity or DNA binding. Only combination of mutants resulted in defects. However, some of these conserved residues have already been implicated in dsDNA binding in various organisms (particularly by the Hofpner lab). Figure 5: It is also well known that the ATPase activity of Rad50 is necessary for the removal of Spo11 and meiotic progression. Although the identified 3KA mutant was not described before, the impact of disrupted ATPase is known. The manuscript would profit from shortening and focus on novel insights."

The authors replied: "It is not well known that Rad50 ATPase activity is required for Spo11 removal. We are unaware of any studies that reveal the significance of ATPase activity for Spo11 removal. I consulted my colleague Scott Keeney who discovered Spo11 and he was also unaware of such studies. No one has tested mutants with defects in ATPase activity per se. We did however learn that mutants we previously showed are defective in Spo11 removal (rad50-46 and others) turn out to have ATPase defects, as we report in this study. With respect to the DNA binding mutants, we were careful to point out that the residues we identify as governing DNA binding have been previously identified and we point out that they were analyzed in charge reversal mutants. We carried out Alanine substitutions. It is a fair criticism that we did not massively revise the interpretations of DNA binding in our study, but we feel that the data do contribute additional substance."

The authors are correct (my apologies), Rad50 ATPase was not per se demonstrated to be required for Spo11 removal, however the role of the ATPase in meiosis is established; e.g. from (PMID: 11371344): "Mutations in the yeast Rad50 nucleotide binding motif have a null phenotype in DSB repair and meiosis, suggesting ATP binding and/or hydrolysis is essential in vivo". With respect to the homodimerization of Rad50 and the involvement of the ATPase domain, the authors should at least cite PMID: 28369545 that was focused on this point.

Reviewer #2

(Remarks to the Author)

The reviewers have addressed my concerns.

Reviewer #3

(Remarks to the Author)

I am happy with the authors responses and the work supports the overall claims of the manuscript with limitations well recognised.

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REVIEWER COMMENTS

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It's clear that ATP hydrolysis is required for access to the Mre11 active site. It's also clear that the closed form (not properly called the "inactive form") engages DNA and subsequently activates Tel1. So, it is very likely that the structure obtained is a bona fide intermediate, likely to be the form required for Tel1 activation. Indeed KP Hopfner, in Rojowska et. al. (PMC4282560) show that the ATP bound form of Rad50 bound to DNA promotes telomere maintenance, which reflects the activation of Tel1.

- The authors identified and described the interface between Mre11 and Rad50, and designed a number of mutant combinations to validate the structure. Some of the mutations however disrupted Mre11 homodimerization, as well as interaction with Xrs2 (bottom part of Fig. 2). I am puzzled by this analysis: the mutations at this point do not validate the structure, what do they serve for? Mutations disrupting interactions with Xrs2 may disrupt nuclear import of Mre11-Rad50 and further complicate interpretation of CPT sensitivity experiments. The rationale of the experiments is not always clear.

The construction and analyses of those mutants was carried out in service of functionally validating the interface structures revealed by cryoEM. WRT mutants that disrupt Xrs2 interaction, there is no ambiguity in our interpretation of CPT sensitivity. We show that those mutants phenocopy null mutations.

- We learn very little about the function of the Mre11 complex. For example: Figure 3: It is well known that homodimerization of Rad50 occurs also through the ATPase domain. Figure 4: The authors observed multiple contacts between Rad50 and dsDNA. Mutating these residues individually had little impact on the protein function as scored by CTP sensitivity or DNA binding. Only combination of mutants resulted in defects. However, some of these conserved residues have already been implicated in dsDNA binding in various organisms (particularly by the

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Provide reference for: "It is well established that defects in dsDNA binding by Rad50 confer sensitivity to clastogenic insult."

This has been demonstrated in multiple publications: Rojowska et. EMBO J (2014) 33: 2847 – 2859. Kissling et. al. Nat Commun 13, 2374 (2022). Seifert EMBO J (2016) 35: 759 – 772. Liu EMBO J (2015) 35: 743 – 758. Chen et. al., JBC DOI 10.1074/jbc.M410192200. Hohl et. al., NSMB 18. 1124-31

In many but not all cases, decrement in DNA binding is linked to a defect in ATP binding.

Reviewer #2 (Remarks to the Author):

In this study, the authors report a 3.2 angstrom resolution cryo-EM structure of the *Saccharomyces cerevisiae* Mre11-Rad50 complex with bound double-stranded DNA (dsDNA). Based on the structure, residues at protein interfaces (homo and heterotypic), and of Rad50 in contact with dsDNA were systematically mutated and tested for DNA damage resistance, complex formation, DNA binding and ATPase activity. The authors identified a single Rad50 residue that is critical for function at the interface with the Mre11 HLH domain, whereas multiple mutations at the interface with the Mre11 capping domain conferred only mild DNA damage sensitivity. Interestingly, they found that the interface between the Mre11 capping domain and Rad50 is important for assembly/stability of the MRX complex. Not too surprisingly, mutation of single residues that contact dsDNA did not confer a phenotype or only mild CTP sensitivity, but combinations of these mutations reduced DNA binding and DNA damage resistance. The authors also identify three lysine residues of Rad50 that are near the dsDNA binding surface and are critical for Mre11 nuclease and ATPase activities. The authors suggest that these residues could be important for transition from the closed to open complex. Analysis of suppressors of the rad50-3KA mutant support the idea that the Rad50 coiled coil domains influence the enzymatic activities of the globular Rad50 head domain.

This is a comprehensive study that provides new insight into the role of conserved residues of Mre11-Rad50 that are critical for function and are frequently mutated in cancers.

Comments:

1. Was Xrs2 interaction with Mre11 tested in the rad50-D1126A mutant?

We repeated the CoIP with rad50-D1126A/N/E mutants, and included also rad50-W1157A/Y and mre11-MRI-6A and MRX null mutants as controls and included the data for this Reviewer. We noted previously (Fig.3D) that Mre11-protein levels were reduced in rad50-D1126A and rad50-W1157 mutants due to the impaired Rad50-Mre11 interactions. We find that both rad50 mutant classes also have reduced Xrs2 protein levels (in Xrs2- and Mre11-IPs), showing that both Mre11 and Xrs2 proteins are destabilized. Thus, Rad50 plays a crucial role in positioning Mre11 for proper interaction with Xrs2.

2. Line 219, “Rad50-D1126 of the HLH domain”? Isn't the HLH domain in Mre11?

Thanks for spotting this mistake. We corrected it.

3. It would be important to measure the ATPase activity of the Rad50-P168S single mutant to see if it is higher than WT. It is possible that this mutation increases ATPase activity independently of the -3KA and -46 mutations.

4. The E356K mutation is the best suppressor of rad50-3KA CPT sensitivity. Was this mutation tested for suppression of the rad50-3KA ATPase activity?

Thanks for these great suggestions. We made MR(P168S) and MR(3KA-E356K) constructs and expressed and repurified MR(WT), MR(EQ), MR(P168S), MR(3KA-E356K) and MR(3KA-P168S) and measured ATPase activities in triplicate (data included for this Reviewer).

The MR(P168S) mutant shows ATPase activity comparable to MR(WT), and thus is not just bypassing rad50-3KA and rad50-46 ATPase defects. The mechanism of this rescue is not clear, but is likely mediated by P168S ability to alter the Rad50-3KA dsDNA interaction to promote ATPase activation by compensating for the loss of the rad50-3KA residues. Based on MD simulation we predicted that P168S suppression should be dependent on K1181 (Fig.6 D). Indeed, rad50-3KA-P168S suppression was dependent on both K1181 and K1183 residues, within the beta-hairpin loop interacting with dsDNA via the minor groove (data not included in manuscript).

Both MR(3KA-E356K) and MR(3KA-P168S) rescued the ATPase activity substantially in comparison to MR(EQ), which phenocopies MR(3KA), but the ATPase activities were not complete, as they did not reach MR(WT) levels.

Also, does it suppress the rad50-46 phenotype?

Very good question. We assessed this prior to the submission but did not include it due to space limitations in the manuscript. The answer is no, P168S is unique as this was the only one which suppressed both rad50-3KA and rad50-46. We included the data for this Reviewer.

5. Line 405. Are authors suggesting that the threshold of ATPase activity is lower for Mre11 nuclease activation than for Tel1 activation?

That's one evident possibility that full ATPase activity might be needed for Tel1 activation but not for Mre11 nuclease. However, as MR(3KA-E356K) shows substantial ATPase activity, but is still deficient in Tel1 activation, this possibility appears now less likely. The rad50-3KA-Sup mutants are in this regard very interesting separation of function mutants promoting Mre11 nuclease but not Tel1 activation. More work will be needed to start to understand how MRX activates Tel1. Some published data (including some of ours) strongly suggest that Rad50 is primarily responsible for Tel1/ATM activation.

Reviewer #3 (Remarks to the Author):

Hohl and colleagues present a 3.2 Å cryo-EM structure of the *S. cerevisiae* Mre11-Rad50 complex with bound dsDNA, representing a closed form, in addition to mutagenesis and functional analysis. They reveal new features of the Rad50-dsDNA interaction interface, and inter- intra protein contacts important for assembly and dynamics.

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Figure 1B – Might be easier to view if the two protomer of Rad50 and MRE11 are slightly different shades of blue and pink (to match to the domain scheme in Figure 1A) rather than using completely different colours for the protomers, it is a little confusing.

Thank you for this comment. We agree and have now colored Mre11 protomers in dark and light orange and Rad50 protomers in light and dark green in the revised version

Why was 83-mer double stranded DNA (dsDNA) chosen?

We choose this substrate as we used it in this and previous publications for EMSA's. We tried different substrates (Splayed arm and hairpin), however we did not get useful data on that.

Page 6- Last paragraph. Could the authors please clarify/expand when they mention they were unable to obtain useable MRX particles? Do they believe they were too heterogenous, flexible or aggregated? Also what is meant by “confounding effects” of Xrs2?

We tried to get cryo-EM structures of MR(WT)X and MR(EQ)X. One major issue was that the Mre11 complex yield of purified complexes in presence of Xrs2 was about 10-20 fold lower than MR alone, so it was more difficult to get a high quality prep.

Further, the particles in the cryo-EM data sets appeared very heterogeneous and perhaps flexible, given that we were unable to obtain high quality 2D class averages for further processing.

Nevertheless, the new human MRN structure from Hopfner lab (bioRxiv 2025.03.14.643254) shows that a segment of bound NBS1 was traceable, with the majority of the protein disordered. In addition, this result showed that the NBS1 does not alter the MR-part of the structure.

We have removed the term “confounding effects”.

NCOMMS-24-75422-A Response to Reviewers

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To address this point, we followed the reviewer's suggestion by adding a clarifying statement to the Results section (p.10) and citing the relevant publication from the Symington lab.

With respect to the novelty, I raised the following points: "We learn very little about the function of the Mre11 complex. For example: Figure 3: It is well known that homodimerization of Rad50 occurs also through the ATPase domain. Figure 4: The authors observed multiple contacts between Rad50 and dsDNA. Mutating these residues individually had little impact on the protein function as scored by CTP sensitivity or DNA binding. Only combination of mutants resulted in defects. However, some of these conserved residues have already been implicated in dsDNA binding in various organisms (particularly by the Hofpner lab). Figure 5: It is also well known that the ATPase activity of Rad50 is necessary for the removal of Spo11 and meiotic progression. Although the identified 3KA mutant was not described before, the impact of disrupted ATPase is known. The manuscript would profit from shortening and focus on novel insights."

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We agree that *in vivo*, Rad50 ATPase activity is essential for all functions of the Mre11 complex, as mutations that disrupt either ATP binding or hydrolysis result in a *rad50Δ*-like phenotype. Based on studies from the Alani and other labs, defects in Rad50 ATP binding likely impair the formation of meiotic DSBs, whereas partial loss of ATPase activity does not block DSB formation but instead interferes with subsequent DSB end processing. The *rad50-3KA* and *rad50-46* mutants are particularly informative in this regard, as they selectively impair meiotic DSB processing without affecting DSB formation. We believe this observation contributes a novel aspect to the current understanding.

Regarding the two suggested citations, we respectfully disagree with their inclusion, as they do not appear directly relevant in the context of our findings.

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Thank you for your valuable review comments which helped us to improve our manuscript.

Reviewer #3 (Remarks to the Author):

I am happy with the authors responses and the work supports the overall claims of the manuscript with limitations well recognised.

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