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

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Abstract

The Mre11–Rad50–Nbs1 (MRN) complex is a central factor in the repair of DNA double-strand breaks (DSBs). The ATP-dependent mechanisms of how MRN detects and endonucleolytically processes DNA ends for the repair by microhomology-mediated end-joining or further resection in homologous recombination are still unclear. Here, we report the crystal structures of the ATP γ S-bound dimer of the Rad50^{NBD} (nucleotide-binding domain) from the thermophilic eukaryote *Chaetomium thermophilum* (Ct) in complex with either DNA or CtMre11^{RBD} (Rad50-binding domain) along with small-angle X-ray scattering and cross-linking studies. The structure and DNA binding motifs were validated by DNA binding experiments *in vitro* and mutational analyses in *Saccharomyces cerevisiae* *in vivo*. Our analyses provide a structural framework for the architecture of the eukaryotic Mre11–Rad50 complex. They show that a Rad50 dimer binds approximately 18 base pairs of DNA along the dimer interface in an ATP-dependent fashion or bridges two DNA ends with a preference for 3′ overhangs. Finally, our results may provide a general framework for the interaction of ABC ATPase domains of the Rad50/SMC/RecN protein family with DNA.

Keywords DNA repair; homologous recombination; microhomology-mediated end joining; protein DNA complex; X-ray crystallography

Subject Categories DNA Replication, Repair & Recombination; Structural Biology

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Introduction

DNA double-strand breaks (DSBs) threaten genome stability in all kingdoms of life and can lead to cell death or gross chromosomal aberrations. They arise during replication fork collapse or exposure to ionizing radiation, reactive oxygen species (ROS), or genotoxic chemicals (Sutherland *et al*, 2000; Costanzo *et al*, 2001; Pommier *et al*, 2003; Mahaney *et al*, 2009). DSBs are also enzymatically generated intermediates in meiotic recombination, V(D)J, and class

switch recombination as well as yeast mating-type switching (Gapud & Sleckman, 2011; Haber, 2012; Xu *et al*, 2012; Lam & Keeney, 2015).

Double-strand breaks can be repaired by different pathways. In canonical non-homologous end-joining (c-NHEJ), the DNA ends are ligated directly in a reaction that depends on Ku and ligase IV. However, a Ku- and ligase IV-independent end-joining pathway also exists and is denoted as either alternative NHEJ (alt-NHEJ) or microhomology-mediated end-joining (MMEJ), since the DNA ends are often processed and joined at microhomologies. All of these template-independent end-joining reactions can result in loss or alteration of genetic information (Chiruvella *et al*, 2013).

The homologous recombination (HR) pathway repairs DNA ends in a typically error-free manner. HR shares the initial DNA processing steps with MMEJ (Truong *et al*, 2013), but the DNA ends are further resected to produce 3′ single-strand tails for homology search and template-dependent extension. The choice between NHEJ and HR is regulated by the cell through multiple mechanisms, in particular the initiation of end resection (Chiruvella *et al*, 2013; Panier & Boulton, 2014; Symington, 2014).

The Mre11–Rad50–Nbs1 complex (and its archaeal Mre11–Rad50, bacterial SbcC–SbcD, and bacteriophage gp46–gp47 homologs, collectively denoted MRN or MR) is a central factor in processing DNA DSBs and hairpins (Stracker & Petrini, 2011; Schiller *et al*, 2014). The complex possesses an ATP-dependent endo/exonuclease activity that processes DNA ends in HR and MMEJ. Specifically, it displays 3′–5′ dsDNA exonuclease activity, hairpin opening activity, ssDNA endonuclease activity and an ATP-dependent dsDNA endonuclease activity (Paull & Gellert, 1998; Connelly *et al*, 1999; Hopfner *et al*, 2000a; Trujillo & Sung, 2001; Truong *et al*, 2013; Cannavo & Cejka, 2014). In the latter, MRN (denoted Mre11–Rad50–Xrs2; MRX in *Saccharomyces cerevisiae*) cleaves the 5′ strand approximately 15–25 nt away from the DSB in a reaction that also requires the Sae2/CtIP protein in eukaryotes (Cannavo & Cejka, 2014). The nuclease activity of MRN helps to clear blocked or modified DNA ends and generates initial 3′ overhangs for MMEJ or further resection in HR (Garcia *et al*, 2011; Langerak *et al*, 2011; Truong *et al*, 2013; Cannavo & Cejka, 2014). MRN also recruits other repair factors to DSBs and helps to elicit the DNA damage response by activating the ataxia telangiectasia mutated (ATM) kinase (Lisby *et al*, 2004; Berkovich *et al*, 2007; Limbo *et al*, 2007; Mimitou & Symington, 2008; Deshpande *et al*, 2014).

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The MRN complex consists of two subunits of the nuclease Mre11, two subunits of the ATPase Rad50, and either one or two Nbs1 subunits (Lammens *et al.*, 2011; Lim *et al.*, 2011; Möckel *et al.*, 2012; Schiller *et al.*, 2012). Nbs1 is only found in eukaryotes and is important for ATM recruitment and activation, nuclear localization of MRN, and recruitment of additional repair factors (Desai-Mehta *et al.*, 2001; You *et al.*, 2005; Limbo *et al.*, 2007; Chen *et al.*, 2008; Mimitou & Symington, 2008). Mre11 together with the Rad50 nucleotide-binding domains (NBDs) and the Mre11-interacting motif of Nbs1 forms the globular catalytic domain of MRN that binds and processes DNA in an ATP-dependent manner. Rad50 further possesses a 15–50-nm-long coiled-coil domain with a terminal Zn-hook dimerization motif (Hopfner *et al.*, 2002; Schiller *et al.*, 2014). The precise mechanistic functions of the coiled-coil domains are still unclear, but they play an important role in NHEJ, ATM activation, and DNA end processing (Hohl *et al.*, 2011; Lee *et al.*, 2013; Roset *et al.*, 2014).

The exact interplay of ATP-dependent DNA binding, processing, and end bridging by MRN is still unclear. Rad50 ATP binding but not hydrolysis is important for checkpoint activation, DNA end bridging, and telomere maintenance, whereas the ATP hydrolysis step is required for DSB repair and DNA processing (Lee *et al.*, 2013; Deshpande *et al.*, 2014; Rojowska *et al.*, 2014). To reveal how MRN interacts with DNA in the presence of ATP—the state that appears to be critical at least transiently for all activities—we determined crystal structures of the *Chaetomium thermophilum* CtRad50^{NBD} dimer in complexes with ATP γ S and DNA or the Rad50-binding domain of Mre11 (Mre11^{RBD}). Our structures clarify how Rad50 binds to DNA in an ATP-dependent fashion by forming a composite DNA binding surface that recognizes approximately 18 base pairs. Fluorescence anisotropy measurements suggest that the ATP-bound Rad50 dimer can also bind two cohesive DNA ends instead of a continuous duplex and has a preference for 3' tails. These structures arise, for example, at telomeres and during HR or MMEJ. Finally, functional studies indicate that the observed DNA interaction is a critical intermediate in DNA end processing. Together, our studies establish a framework for the architecture and ATP-dependent dynamics of the eukaryotic Mre11–Rad50 catalytic head module and reveal how Rad50 binds dsDNA and bridges DNA ends in an ATP-dependent manner.

Results

Structure of *Chaetomium thermophilum* Rad50^{NBD} in complex with ATP γ S:Mg²⁺ and the Rad50-binding domain of Mre11

To obtain the structure of a eukaryotic Rad50 protein and its complex with Mre11^{RBD}, we co-purified CtRad50^{NBD} with the putative Rad50-binding domain of Mre11 (Appendix Fig S1). Crystals containing a Rad50^{NBD} dimer bound to two Mre11^{RBD}s (residues 438–531) and two ATP γ S:Mg²⁺ molecules in the asymmetric unit diffracted to 3.0 Å and we obtained experimental phases by a single-wavelength anomalous diffraction experiment with selenomethionine-derivatized protein. Data collection and refinement statistics are summarized in Appendix Table S1.

Two CtRad50^{NBD}s assemble into a dimer with two ATP γ S:Mg²⁺ molecules sandwiched in the dimer interface by the opposing

Walker A, Walker B, and signature motifs (Fig 1A and B) (Hopfner *et al.*, 2000b). In general, our structure represents a pre-hydrolysis state with a formed catalytic “dyad” between Glu1238 (Walker B motif) and His1275 (His-switch) (Zaitseva *et al.*, 2005). The two protruding coiled-coil domains each bind one Mre11^{RBD}, which is a five-membered helical bundle that predominantly interacts with the C-terminal α -helix of the antiparallel Rad50 coiled-coil domain (Fig 1C). The first three α -helices of Mre11^{RBD} bind the coiled-coil, the short fourth helix caps the RBD, while the fifth helix forms a “spine” that protrudes backwards to the lobe I of Rad50^{NBD}. The structure of eukaryotic Mre11^{RBD}–Rad50^{NBD} generally resembles that of its prokaryotic homologs, but it displays notable differences and extensions (Fig 2).

Perhaps the most unexpected of these differences is the substantially enlarged Rad50-binding domain of Mre11. RBDs of bacterial SbcD (*Thermotoga maritima*) and archaeal Mre11 (*Methanocaldococcus jannaschii*) correspond to α -helices 1–2 or 1–3 of the RBD of CtMre11. The location of the CtMre11^{RBD} helix α 5, pointing toward the Rad50^{NBD}, suggests that the remaining C-terminal polypeptide chain of eukaryotic Mre11 (approximately 100–200 additional amino acids depending on the species) is situated in the vicinity of the globular “head” of MRN, consistent with findings that identify the C-terminal region as important for stable DNA and Xrs2 binding as well as for meiotic recombination (Furuse *et al.*, 1998; Usui *et al.*, 1998; Bhattacharyya *et al.*, 2008). Another indication of the importance of the conformation of the Mre11^{RBD} domain is the fact that a mutation in this domain (T481→K in human) was found in two patients with AT-like disease (ATLD5/6) (Delia *et al.*, 2004). Human T481 corresponds to Q489 in *C. thermophilum*, which is located in helix α 3 in CtMre11^{RBD} (Figs 1 and 2). It is likely that the mutation affects the stability of the RBD and the interaction between the RBD and the Rad50 coiled-coil.

A further noteworthy difference is insertion II, which is located in close proximity to the ATP-coordinating residues 62–68 (Figs 2 and EV1) and forms a short α -helix at the Rad50–Rad50 interface in the ATP-bound state. Interestingly, the regions around insertions I and II harbor three Rad50S mutations (Ser14→Pro, Arg20→Met, and Val63→Glu in *S. cerevisiae*), suggesting that these insertions could play a role in the regulation of MRN activity by CtIP/Sae2 (Alani *et al.*, 1990; Cannavo & Cejka, 2014). In particular, Ser14 and Ala64 (corresponding to Ser14 and Val63 in *S. cerevisiae*) contact ATP via main chain interactions and are located at the Rad50–Rad50 dimer interface, suggesting that ATP-dependent engagement of Rad50^{NBD} or ATP hydrolysis is compromised (Appendix Fig S2). Compared with prokaryotic Rad50, the CtRad50 dimer groove is enlarged by β -hairpin insertion III on top of lobe I and by insertion IV, a β -hairpin (β 8 and β 9) that binds along the coiled-coil.

Architecture and dynamics of the eukaryotic Mre11–Rad50 head complex

The structure of CtMre11^{RBD}–Rad50^{NBD} reported here together with a structure of the catalytic domain dimer of CtMre11 (CtMre11^{CD}) (Seifert *et al.*, 2015) enabled us to address the architecture and dynamics of the eukaryotic Mre11–Rad50 head module by chemical cross-linking and mass spectrometry (CXMS) in combination with small-angle X-ray scattering (SAXS). We superimposed the crystal structures of CtMre11^{CD} and CtMre11^{RBD}–Rad50^{NBD} as rigid bodies

Figure 1. Structure of the *Chaetomium thermophilum* Mre11^{RBD}–Rad50^{NBD} complex.

A Ribbon representation with highlighted secondary structure of the Mre11–Rad50-binding domain (CtMre11^{RBD}; light and dark blue) bound to the nucleotide-binding domain (NBD) of Rad50 (CtRad50^{NBD}; light and dark orange). Two ATPγS:Mg²⁺ (magenta and green, respectively) molecules are bound in the CtMre11^{RBD}–Rad50^{NBD} dimer interface. The red sphere in the Mre11^{RBD} denotes the corresponding location of the human ATLD5/6 mutation.

B Stereo view of the ATP binding site. The ATP-interacting residues are highlighted and selected hydrogen bonds are indicated.

C Detailed view of the CtMre11^{RBD}–Rad50^{NBD} interface. Residues that mediate contacts between the CtMre11^{RBD} and the coiled-coil (CC) domain of Rad50 are represented as sticks.

onto the crystal structure of archaeal Mre11–Rad50^{NBD} (PDB code 3AVO), resulting in a very reasonable fit between the Mre11 dimer and the Rad50 dimer (Fig 3A). In this modeled complex, the C-terminus of the Mre11 capping domain (Ala412) and the N-terminus of Mre11^{RBD} (Ser438) are approximately 10 Å apart, a distance that could be easily spanned by the 25 amino acids that connect these modules in the primary structure.

To validate this model, we cross-linked the MRN head complex (MRN^{hc}) with the lysine-specific cross-linker disuccinimidyl suberate (DSS) in the presence and absence of ATPγS:Mg²⁺ and identified

cross-linked peptides by mass spectrometry (Appendix Fig S3) (Tosi et al, 2013; Leitner et al, 2014). Cross-links were found between all three different polypeptide chains (Fig 3B): 91 specific non-redundant cross-links in the presence and 149 non-redundant cross-links in the absence of ATPγS:Mg²⁺. The C-terminal part of the Nbs1 construct used here cross-links with many regions of the Rad50^{NBD} and Mre11 and is probably flexible. Next, we mapped cross-links between Mre11 and Rad50 onto the model for the closed complex. In the presence of ATPγS, we identified 15 cross-links between Mre11 and Rad50. All cross-links, except two identified in

Figure 2. Comparison of eukaryotic and prokaryotic Mre11^{RBD}–Rad50^{NBD}.

A–D Crystal structures of *Chaetomium thermophilum* (A), *Pyrococcus furiosus* (B), *Thermotoga maritima* (C), and *Methanocaldococcus jannaschii* (D) Mre11^{RBD}–Rad50^{NBD} complexes are shown (PDB codes: 5DA9, 3QKU, 3QF7, and 3AV0, respectively). Eukaryotic-specific expansions in Mre11^{RBD} (dark blue) and Rad50^{NBD} (orange) are highlighted. Of note is the substantial enlargement of Mre11's RBD compared to the prokaryotic orthologs.

the presence of ATP γ S:Mg²⁺, map with a lysine C α –lysine C α distance of 15–41 Å, validating the docked model (Fig 3C). In the absence of ATP γ S, we identified 35 cross-links between Rad50 and Mre11. The increased number of cross-links could be the result of an increased flexibility between Mre11 and Rad50 or the presence of additional conformational states. In support of these possibilities, we also find a much broader distance distribution of these cross-links when mapped onto the model for the closed conformation, with many cross-links mapping to C α –lysine C α distance of > 41 Å.

To further analyze ATP-dependent structural dynamics, we performed SAXS analyses. Both the maximum distances (Dmax) and the mean distances in the particle become substantially smaller in the presence of ATP γ S (Fig 3D). We have not been able to model the complex on the basis of the SAXS data in the absence and presence of ATP γ S; presumably, we still have subpopulations of different states or have highly flexible coiled coils. In addition,

the scattering curves indicate that CtMR does not adopt a stable “open” form as has been observed for the bacterial complex. Nevertheless, our data suggest that, similar to bacterial and archaeal MR, ATP also induces a more closed state in eukaryotic MR (Lammens *et al*, 2011; Williams *et al*, 2011; Möckel *et al*, 2012; Deshpande *et al*, 2014).

Taken together, these analyses show that the eukaryotic Mre11–Rad50 head module undergoes ATP-dependent structural transitions and can adopt a more compact state in the presence of ATP, consistent with the model that a Rad50 dimer binds into the active site groove of the Mre11 dimer.

Structural basis for ATP-dependent DNA binding by Rad50

To establish a framework for DNA binding to eukaryotic Rad50 and to reveal how ATP promotes DNA binding to Rad50 proteins, we

Figure 3. Architecture and dynamics of the Mre11–Rad50^{NBD} head module.

A Comparison of bacterial, archaeal, and eukaryotic MR^{NBD} in the ATP-bound state. Mre11 N- and C-termini as well as the Rad50 coiled-coil (CC) domains are marked. Eukaryotic MR^{NBD} docking model consists of dimeric CtMre11^{CD} (light and dark blue) and CtMre11^{RBD}–Rad50^{NBD} (Mre11 in light and deep blue; Rad50 in beige and orange) superimposed onto MR^{NBD} (PDB code: 3AV0) from *M. jannaschii*. Identified cross-links between the Rad50 and Mre11 subunits are marked as green lines.

B Map of the identified intra (red) and inter-protein cross-links (gray) between the MRN subunits.

C Distribution of the measured lengths of the cross-links in the ATP-bound CtMR^{NBD} model. Data from protein samples with and without ATPγS are depicted.

D SAXS scattering curve (left) and P(r) distribution curve (right) of MR^{hc} (Appendix Fig S1) with (blue) and without (red) ATPγS:Mg²⁺ show a compaction of the complex upon binding of ATPγS:Mg²⁺.

Source data are available online for this figure.

crystallized CtRad50^{NBD} in the presence of a 22mer dsDNA and ATP γ S:Mg²⁺. Crystals in space group P2₁2₁2₁ diffracted X-rays to 2.5 Å resolution and we determined the structure by molecular replacement using CtRad50^{NBD} as a search model. The asymmetric unit contained one Rad50 dimer bound to two ATP γ S:Mg²⁺ molecules and 15-bp dsDNA. Although stoichiometric amounts of Mre11 were also present in the crystallization drops, Mre11 was not part of the crystals. Data collection, refinement, and model statistics are summarized in Appendix Table S1.

DNA is well defined in the electron density and forms a quasi-continuous, undulating B-form DNA double helix in the crystal lattice (Figs 4A and EV2A and B). The asymmetric unit accommodates only 15 of the 22 base pairs, suggesting that either Mre11 in the crystallization drops partially degraded the DNA during the long crystallization time (4 months) or the DNA molecules are shifted between adjacent asymmetric units. For that reason, we were unable to assign the sequence of the co-crystallized DNA to the DNA model. However, density for the DNA backbone and bases is for the most part well defined and we consequently modeled bases using a generic oligo(dG:dC) 15mer into the electron

density, which improved the stereochemistry, *R*-values, and the DNA density.

The DNA duplex is situated in the positively charged groove between the two coiled coils of the Rad50 dimer (Figs 4B and EV2C–F). Each of the two DNA strands binds to both sides of the Rad50 dimer in a pseudo-symmetric fashion. The observed DNA binding mode explains the previously unclear dependency of Rad50 DNA binding on the presence of ATP: The ATP-driven reorientation of lobes I and II, and dimerization of two Rad50^{NBD}s positions assemble ten DNA binding motifs (five on each side of the dimer) to recognize an approximately 18-base pair-long DNA duplex via both backbone strands.

Details of ATP-dependent DNA binding of the Rad50 dimer

The five DNA binding motifs are short amino acid sequences or single amino acids along the groove of the Rad50 dimer and together they bind in total 12 bases within the 18-bp duplex (Fig 5A–C). The DNA is recognized through the minor groove backbone in a sequence-independent manner, whereby motifs I–III and

Figure 4. CtRad50^{NBD}–DNA crystal structure.

- A The DNA forms a quasi-continuous helix in the crystal lattice. Shown are two adjacent Rad50^{NBD} dimer complexes along with the 2mF_o–DF_c density (blue) contoured at 1 σ for the dsDNA (light and dark orange). The quasi-continuous DNA argues against a role of the Rad50 DNA binding module in direct DNA end recognition.
- B Cartoon model of the dsDNA (teal) in complex with the ATP γ S:Mg²⁺-bound Rad50^{NBD} dimer (light and dark orange). DNA binds to both halves of the Rad50^{NBD} dimer in a pseudo-symmetric fashion, revealing the mechanistic link between ATP-dependent dimerization of the Rad50^{NBD}s and dsDNA binding.

motifs IV–V clamp the DNA between the opposing Rad50 protomers. For subsequent discussion, we will denote the strand polarity as the direction from the center toward the outside of the Rad50 dimer, that is, a 3′→5′ strand on one side of the Rad50 dimer becomes the 5′→3′ strand on the other side and vice versa.

Motif I is the top strand of the peripheral β -sheet (β_6) of the ATP binding cassette (ABC) fold and binds the 5′→3′ strand via interactions between the backbone of two consecutive bases to the main chain oxygen atoms of Thr110 and Gln113 as well as the main chain nitrogen atom of Thr113 (Fig 5A–C). Motif II, the previously identified strand-loop-helix motif (Rojowska et al, 2014), contributes to the DNA interaction by providing charge complementarity and through interactions of Arg132 with the major groove and/or DNA backbone, but intriguingly appears to have a minor role in overall DNA recognition compared to what was previously found for bacterial Rad50 (see Discussion).

The 3′→5′ strand is bound across the Rad50^{NBD} dimer interface by motifs III, IV, and V. Motif III is situated in the central cavity of the DNA binding groove and connects the two main ATP binding elements, the helix α_A (following the P-loop/Walker A motif) and the adenine recognition loop (aa 64–68) (Fig 5A–C). As such, this loop could play an important role in coupling DNA binding and ATP binding or hydrolysis. Motif III binds a DNA backbone phosphate via main chain and side chain interactions with Asn58 and by inserting Arg61 into the minor groove. The two preceding phosphates are recognized by Arg1204 (motif V) and motif IV from the opposing NBD. Motif IV is located at the N-terminal turn of α_F , which connects the nucleotide-binding and coiled-coil domains. It is located at the N-terminal end of the “signature coupling helix” as previously defined (Williams et al, 2011). Arg1204 (motif V) is situated in the Rad50 dimer interface and, in addition to directly binding to the phosphate backbone, it also stacks with Asn58 on motif III and could consequently more broadly facilitate DNA binding.

In summary, both strands of the dsDNA are recognized in a fashion that predominantly involves hydrogen bonds between the DNA backbone and the protein main chain in conjunction with three arginine fingers that reach into the minor groove or directly bind the DNA backbone at the Rad50 dimer interface. The interactions with Arg1204 and motifs III and V can only form in the tightly engaged, ATP-bound Rad50^{NBD} dimer, which provides a further link between ATP and DNA binding to Rad50.

Functional analysis of Rad50 DNA interaction in *Saccharomyces cerevisiae*

To test the relevance of the observed ATP-dependent DNA interaction of Rad50 in a functional context *in vivo*, we analyzed the capability of *rad50* mutants to rescue the camptothecin (CPT, topoisomerase I inhibitor) sensitivity of a $\Delta rad50$ strain of *S. cerevisiae*. The Rad50^{NBD}–DNA structure suggests that disruption of the dimer should abolish the ability of Rad50 to bind dsDNA and consequently hinder DNA repair. In agreement with this prediction, the dimer interface mutant S1205^{Sc}→R (corresponding to Ser1208^{Ct}), which prevents ATP-induced engagement of two Rad50^{NBD}s (Fig EV3), shows high CPT sensitivity and displays the same phenotype as the $\Delta rad50$ (Fig 5D; Appendix Fig S4A). To investigate whether the Rad50 mutants show CPT sensitivity purely because of their compromised DNA binding ability, or whether Rad50 dimerization

is also necessary for other functions of the complex, we tested both the charge-reversal mutant K60^{Sc}→E (corresponding to Arg61) in motif III and the double mutant K60^{Sc}→E R131^{Sc}→E. Arg61 binds the minor groove of dsDNA, but the mutation does not disrupt the dimerization of Rad50 (Fig EV3). Furthermore, DNA binding mutations do not disturb the overall integrity of the MRX complex in *S. cerevisiae* (Appendix Fig S4B). K60^{Sc}→E also shows CPT sensitivity, indicating that the phenotypes of the tested mutants result from their compromised ability to bind DNA (see below). We therefore conclude that for the repair of CPT-induced DNA damage, residues involved in ATP binding and hydrolysis, and consequently promote dimerization and formation of the DNA binding groove, play an equally important role as those that directly interact with the DNA.

In a previous study, we tested a number of mutants based upon the crystal structure of *Thermotoga maritima* (Tm) Rad50 in complex with dsDNA. The mutant R1201^{Sc}→E (corresponding to R1204^{Ct}) displayed severe defects in both telomere maintenance and DSB repair, but the basis for this phenotype remained enigmatic because the corresponding residue in TmRad50 does not directly contact the dsDNA in the crystal structure (Rojowska et al, 2014). However, the cause of the phenotype is clear from the structure of CtRad50 because this residue both directly contacts the backbone of the DNA and forms part of the dimer interface.

In summary, these mutational data suggest that interaction of DNA along the Rad50 dimer groove is critical for the repair of DSBs by MRN.

DNA double-strand break bridging

Biochemical studies indicated that MR and to a minor extent Rad50^{NBD} can bridge two DNA ends in the presence of ATP, a function that is important for end-joining (Deshpande et al, 2014). While our structure indicates that the ATP-bound Rad50 does not directly recognize a DNA end, it is possible that Rad50 could bridge two DNA ends by a mechanism that involves stacking of two DNA ends across the Rad50 dimer groove. To address this question, we performed fluorescence anisotropy measurements, which allow the measurements of precise dissociation constants (K_d) (Figs 6 and EV4; Appendix Tables S2 and S3). We first tested the effect of the DNA length as well as the presence of ATP on the DNA binding affinity of the Rad50^{NBD}. A 35mer that could span the entire binding site of the Rad50 dimer was not bound by CtMre11^{RBD}–Rad50^{NBD} in the absence of ATP, but robustly bound with a $K_d = 0.50 \pm 0.02 \mu\text{M}$ in the presence of ATP (Fig 6 and Appendix Table S2). These data show that ATP is critical for DNA binding to CtMre11^{RBD}–Rad50^{NBD} and validate the structural data. A corresponding dsDNA 17mer that is too short to fully reach across the Rad50^{NBD} dimer was bound with a $K_d = 2.33 \pm 0.09 \mu\text{M}$ to ATP–Mre11^{RBD}–CtRad50^{NBD}. This substantially reduced affinity compared to the 35mer DNA is consistent with the structural results that show that Rad50 needs 18 bp to fully reach across its DNA binding platform.

However, instead of binding a single duplex of at least 18 bp, the Rad50 dimer might also bind two DNA ends with either stacked or annealed complementary overhangs. To address this possibility, we also tested DNA substrates with different types of overhangs. A 20mer with a five base 5′ overhang has a similar affinity ($K_d = 2.13 \pm 0.10 \mu\text{M}$) to the blunt-ended 17mer. However, a 5-base pair 3′ overhang resulted in a notable increase in binding

Figure 5. Detailed view on the Rad50–DNA interaction.

A View along the DNA binding groove in a cut-open ribbon representation. One Rad50^{NBD} protomer is shown in full (orange and brown) and the other one is shown in part (beige). DNA binding motifs as identified here are numbered. DNA binds with both strands in a B-form conformation along the dimer groove to five motifs (I–V), predominantly via backbone–main chain interactions. Two notable arginines stabilize the interaction with DNA by interacting with the phosphate backbone (R1204) or binding into the minor groove (R61).

B Scheme of the dsDNA and the interacting residues on only one-half of the Rad50 dimer is shown. The structure was analyzed with PDBsum (<http://www.ebi.ac.uk/pdbsum>) (de Beer et al, 2014) and the scheme was optimized manually. Motifs in protomer A and protomer B are highlighted in beige and orange, respectively.

C Detailed view of the Rad50–DNA interaction (only one-half of the pseudo-symmetric binding site is shown). Motifs are numbered and selected residues are highlighted in stick representation. Note the “sandwich” type of binding of the DNA duplex between motifs I, II, III, R61 (minor groove), and R132 (major groove) on one protomer and motifs IV and V (both backbone) on the other protomer, which can occur only in the ATP-bound engaged Rad50^{NBD} dimer.

D Plate survival assay with wild-type, *rad50Δ*, and *rad50* mutant strains on YPD and DNA damage-inducing camptothecin (CPT) (for details, see Materials and Methods).

affinity ($K_d = 1.25 \pm 0.22 \mu\text{M}$), indicating a preference for 3' overhangs. This distinction can be explained by the binding mode of DNA to the Rad50 dimer (see Discussion). Extending the 3' or 5' overhangs to 20 bases resulted in tight binding with K_d values of $0.36 \pm 0.02 \mu\text{M}$ and $0.59 \pm 0.03 \mu\text{M}$, respectively. These long DNAs could easily span the Rad50^{NBD} dimer, but at least a partial DNA duplex is required since a 35-b ssDNA bound with a reduced $K_d = 1.71 \pm 0.23 \mu\text{M}$.

To test the simultaneous binding of two DNA ends to the Rad50 dimer, we mixed two 20mers that contained five nucleotide long complementary 3' or 5' overhangs. The two 20mers in each of the two mixtures can anneal via these overhangs, resembling two bridged partially processed DNA ends with a short homology. In the case of 5' overhangs, we calculated a $K_d = 1.77 \pm 0.09 \mu\text{M}$ for the labeled DNA in the presence of a second 20mer with a complementary overhang. Thus, the binding affinity is only slightly increased

Figure 6. DNA binding analysis by fluorescence anisotropy.

A–D Data points represent the change in fluorescence anisotropy (arbitrary units). The various DNA ligands are depicted with different colors and symbols (see inset). DNA binding for the 35mer dsDNA is shown in all panels as high-affinity standard. Data are means of three independent experiments, and error bars represent standard error ($n = 3$). The solid lines represent the least square fit to a Hill binding model. DNA binding of wild-type CtMre11^{RBD}–Rad50^{NBD} to 35-bp dsDNA in the presence and absence of ATP as well as to different DNA substrates was tested (A–C). The affinity to 35-bp dsDNA was also measured with wild-type CtMre11^{RBD}–Rad50^{NBD} and CtMre11^{RBD}–Rad50^{NBD} containing Rad50 mutants (R61E, R132E, R61E+R132E, S1208R) (D).

compared to the K_d in the absence of the second 20mer. However, the situation is substantially different in the case of complementary 3' overhangs. Here, we calculated a $K_d = 0.48 \pm 0.04 \mu\text{M}$ for the labeled DNA in the presence of a second molecule with a complementary overhang. This affinity is almost the same as observed for the continuous 35-bp dsDNA ($K_d = 0.50 \pm 0.02 \mu\text{M}$).

In summary, these data provide a quantitative evaluation of ATP-dependent binding of DNA to the Mre11^{RBD}–Rad50^{NBD} module. Consistent with the structural analysis, the equilibrium binding assays suggest that the ATP-bound Rad50 dimer binds either a continuous duplex, a partial duplex of sufficient length, or two DNA ends that are annealed via 3' overhangs.

Discussion

The MR complex is a central factor in the metabolism of DNA ends in all kingdoms of life and has multiple functions in the tethering, processing, and—in eukaryotes—checkpoint signaling of DSBs (Stracker & Petrini, 2011). A critical role of MRN is the ability to process blocked or modified DNA ends in order to elicit MMEJ or HR. These DNA ends include meiotic breaks with covalently attached Spo11, hairpin structures, abortive topoisomerases, or DNA ends bound by Ku (Liu *et al*, 2002; Lobachev *et al*, 2002; Neale *et al*, 2005; Mimitou & Symington, 2010; Langerak *et al*, 2011; Sacho & Maizels, 2011). The yeast ortholog Mre11–Rad50–Xrs2 (MRX) cleaves the 5' strand approximately 15–25 nt from the DNA end *in vitro* in a reaction that requires ATP hydrolysis, Mre11's nuclease motif, and the Sae2/CtIP protein (Garcia *et al*, 2011; Cannavo & Cejka, 2014; Shibata *et al*, 2014). Endonucleolytic cleavage of DNA near the breaks has also been observed for both archaeal and bacterial homologs, and could play a role in deprotection of DNA ends in bacteriophage T4 recombination (Connelly *et al*, 2003; Hopkins & Paull, 2008; Almond *et al*, 2013).

The precise mechanism of how ATP aids and regulates nucleolytic and structural functions in all of these reactions is not understood. Structural studies established that in the presence of ATP, prokaryotic MR complexes adopt a “closed” conformation in which the Rad50^{NBD} dimer binds into the Mre11 active site groove and blocks its nuclease active site (Lim *et al*, 2011; Möckel *et al*, 2012). In contrast, the TmMR complex in the absence of ATP adopted an open state with exposed Mre11 nuclease active sites (Lammens *et al*, 2011), consistent with its ability to act as a 3'–5' exonuclease or open hairpin structures (Paull & Gellert, 1998). The present structure of the *C. thermophilum* ATP-Mre11^{RBD}–Rad50^{NBD} dimer, our recently reported structure of the *C. thermophilum* Mre11^{CD} (catalytic domain) dimer (Seifert *et al*, 2015), and CXMS and SAXS analyses suggest that for the eukaryotic MRN head module ATP leads a closed state similar to what has been observed for the prokaryotic MR head (Fig 7A).

Work by others and us has suggested that the ATP-bound state is critical for telomere maintenance, end-joining, DNA tethering or DNA end bridging, and ATM activation (Nakada *et al*, 2003; Lee *et al*, 2013; Deshpande *et al*, 2014; Rojowska *et al*, 2014). The structure reported here reveals now how DNA is bound to ATP–Rad50. The Rad50 dimer preferentially recognizes > 18 bp of a continuous DNA duplex, as well as a longer duplex DNA with 3' overhangs, or two annealed DNA ends with short complementary 3' overhangs. The structure argues against a direct role of the Rad50 module in the sensing of single DNA ends, a function that we propose resides in the Mre11 module. The Mre11 dimer has previously been shown to bind a single DNA end or (Sung *et al*, 2014) alternatively bridge two DNA ends (Williams *et al*, 2008). Furthermore, the preference for 3' tails by the Rad50^{NBD} is consistent with data on the full-length human MRN. In the absence of ATP, it was observed that human MR has a preference for 5' tails, whereas in the presence of ATP or AMP-PNP, the complex gained a strong preference for 3' tails (de Jager *et al*, 2002). Thus, in the absence of ATP, DNA end bridging could occur via Mre11, which might have a preference for 5' overhangs according to the crystal structures (Williams *et al*, 2008), whereas in the presence of ATP, Rad50 could bridge the resected 3' tails.

In the bridging conformation, DNA with short 3' overhangs is able to interact with its duplex at motifs I and III of one NBD, while the 3' overhang can continue over to the second NBD to interact with motifs IV and V. Therefore, two annealed 3' overhangs would have the nicks at the solvent-exposed side of the bound DNA. In contrast, two short annealed 5' overhangs would have the nicks at the interface of DNA and protein, weakening their interaction (Fig 7B). Along the same arguments, the preference for 3' overhangs might be alleviated for longer complementary overhangs. In any case, by bridging DNA ends with 3' overhangs at microhomologies, arising after MRN- and CtIP-dependent initial processing of DNA, MRN could help to protect the DNA ends from further processing (e.g., by MRN's own ssDNA endonuclease). Since MR is important for the end-to-end coordination of recombination in bacteriophages (Almond *et al*, 2013), such a direct bridging of DNA ends could be a more universal function of the complex as well (Fig 7C).

The DNA binding mode reported here is somewhat different to the one previously observed for the bacterial Rad50–ATP–DNA complex (Rojowska *et al*, 2014). In the bacterial complex, the DNA did not bridge both Rad50 protomers and could not explain why Rad50 requires ATP for robust DNA binding (Fig EV5A–C). Furthermore, the binding motifs on lobe I differ between the bacterial and eukaryotic complexes: In the prokaryotic complex, the 5'–3' strand binds along $\beta 7$, while in the eukaryotic complex, the 3'–5' strand binds along $\beta 6$. Rad50 consequently appears to “hop” from one DNA strand of the duplex to the other between both structures. This binding promiscuity could help Rad50 retain DNA contacts when, for instance, one of the two ATP molecules is

hydrolyzed and the NBD dimer is partially opened up during other conformational changes in the reaction cycle of MRN or MR complexes. Interestingly, we find that the signature motif mutant S1208R prevents ATP-dependent dimerization but still leads to increased DNA binding in the presence of ATP than the wild-type Rad50 in the absence of ATP. This suggests that ATP stabilizes a conformation within the “monomeric” NBD that leads to a higher affinity DNA binding. It has been previously shown that ATP-induced conformational transitions between lobes I and II of *P. furiosus* Rad50^{NBD} regulate the different functions of the complex and that the apo and ATP-bound conformations are stabilized via alternating hydrogen bonding and ion pair networks (Williams *et al*, 2011; Deshpande *et al*, 2014). Although we do not have a structure of apoRad50^{NBD} for a detailed comparative study, we do observe an intricate polar bonding network also in the ATP-bound *C. thermophilum* Rad50^{NBD}, suggesting that a related conformational change could take place upon ATP binding to eukaryotic Rad50. Such a conformational state might increase DNA binding at the monomeric NBD (as deduced from the S1208R mutation) via a proper orientation of the DNA binding motifs on lobes I and II.

Finally, our data establish that recognition of DNA along the groove of ATP-bound Rad50 dimers is also important for the repair functions of the complex, since Rad50 alleles with mutation in the core DNA binding residue K60^{Sc} cannot rescue the CPT sensitivity of $\Delta rad50$. We previously showed that R1201E^{Sc} led to sensitivity against hydroxyurea, CPT, and bleomycin (Rojowska *et al*, 2014), suggesting that DNA binding to Rad50, as observed here, is

Figure 7. Model of the MR complex in DNA end bridging.

- A Composite model of the ATP-bound MRN complex (Rad50 dimer in light and dark orange; Mre11 (PDB code 4YKE) dimer in light and dark blue, Nbs1 (PDB code 4FBW) in magenta) bound to dsDNA (teal). The C-terminal tails of Mre11 and the N- and C-terminal parts of Nbs1 are not part of the structural model and are indicated as dashed lines.
- B Unified model for the ATP-dependent binding of a blocked (gray sphere) DNA end and bridging of two DNA ends by Rad50. Orange and yellow circles represent key interacting DNA backbone phosphates. DNA binding motifs I–V can bind either continuous dsDNA or two end-to-end stacked DNA ends. The particular arrangement of motifs explains the preference of complementary 3' overhangs over blunt ends or 5' overhangs. In the case of 3' overhangs, the two tails can anneal and generate a cohesive bridge without discontinuities at the Rad50 DNA binding motifs.
- C Our results can explain how MRN can bind to both continuous DNA near blocked DNA double-strand breaks but also function in the bridging of DNA ends in end-joining reactions, preferentially those with 3' overhangs and microhomologies.

important for a broad range of lesions that require MRX for repair. Most importantly, the requirement for DNA binding along the Rad50 groove in the ATP-bound conformation argues against a simple two-state “open” and “closed” model for the action of MRN at DNA lesions. How can Mre11 process the DNA in the closed state (Fig 7A)? One possibility is that the Mre11 dimer is structurally remodeled or displaced upon ATP and DNA binding to Rad50 such that Mre11’s nuclease can reach the DNA bound to ATP–Rad50. Although the nature of such a hypothetical remodeling or opening of the Mre11 dimer requires future studies, previous cross-linking data indicate that substantial structural changes occur in the Mre11 dimer in response to Rad50 binding ATP (Lamens *et al*, 2011).

In summary, the structure of the eukaryotic ATP γ S–Rad50–DNA complex reveals the enigmatic mechanism for the ATP-dependent recognition of DNA by Rad50 and provides a framework for a functionally critical DNA state of MRN in DNA processing, end bridging, and signaling. They provide the first structural framework for the ATP-regulated interaction of a chromosome-associated ABC ATPase of the Rad50/SMC/RecN protein family with DNA.

Materials and Methods

Protein preparation

For co-expression of different MR(N) sub-complexes, plasmids were co-transformed into *E. coli* cells and expression was carried out at 18°C overnight. Cells were centrifuged, resuspended in buffer A (25 mM Tris pH 8.0, 300 mM NaCl, 10 mM imidazole), and lysed. Protein was purified using metal-affinity and size-exclusion chromatography. Details about protein synthesis and purification are provided in the Appendix.

Crystallization, data processing, structure determination, and refinement

Crystals of CtMre11^{RBD}–Rad50^{NBD} were grown by hanging drop vapor diffusion. Diffraction data up to a resolution of 3.0 Å were collected, indexed, and scaled (see Appendix Supplementary Methods). The structure of CtMre11^{RBD}–Rad50^{NBD} was solved by single-wavelength anomalous diffraction (SAD). The crystals contained one Mre11^{RBD}–Rad50^{NBD} dimer per asymmetric unit and the space group was I 222.

To crystallize CtRad50^{NBD}–DNA, the purified MR^{hc} (Appendix Fig S1) complex (14.1 mg/ml) was mixed with 12 mM MgCl₂, 5 mM ATP γ S, and 95 μ M dsDNA. After 4 months, crystals appeared were cryoprotected and flash-cooled in liquid nitrogen. Diffraction data up to a resolution of 2.5 Å were collected and the structure was solved by molecular replacement using *Phaser* with the structure of CtRad50^{NBD} as a search model (McCoy *et al*, 2007). After rounds of manual model building and refinement, density in the *mF*_o–*DF*_c electron density map allowed modeling of B-form dsDNA. The space group was P2₁2₁2₁ and Rad50^{NBD}–DNA crystals contained one dimer and 15-bp dsDNA per asymmetric unit. See Appendix for more details and Appendix Table S1 for collection and refinement statistics. Figures were prepared using PyMOL (Schrodinger, 2010).

Small-angle X-ray scattering (SAXS) experiment

Chaetomium thermophilum MR^{hc} protein was purified as described and the flow through of the concentration step was used as buffer reference for the small-angle X-ray scattering (SAXS) measurements. The MR^{hc} complex without ATP γ S was concentrated to 4.4 mg/ml. For measurement of the MR^{hc} complex in the presence of a non-hydrolyzable ATP analog, 2 mM ATP γ S and 8 mM MgCl₂ were added before concentrating the protein up to 4.9 mg/ml. The two samples were measured at the EMBL P12 beamline of the German Electron Synchrotron (DESY, Hamburg, Germany). The SAXS data were analyzed using the ATSAS 2.5.1 package (Petoukhov *et al*, 2012). The P(r) distribution curve shows a smaller D_{max} of the protein in the presence of ATP γ S and the mean inter-atomic distance in the protein also decreases. Details are provided in the Appendix.

Chemical cross-linking experiment and mass spectrometry (CXMS) analysis

The MRN^{hc} complex (Appendix Fig S1) was applied to a S200 GL10/300 (GE Healthcare) size-exclusion chromatography column equilibrated with 200 mM NaCl and 25 mM HEPES pH 8.2. For analysis in the presence of ATP γ S, the protein was mixed with 12 mM MgCl₂ and 5 mM ATP γ S prior to the cross-linking reaction. In short, the protein complex was cross-linked with an equimolar mixture of isotopically light and heavy labeled disuccinimidyl suberate (DSS-d0/d12, Creative Molecules Inc.). The reaction was performed at 30°C for 35 min and quenched with 100 mM Tris–HCl pH 8.0 (final concentration). The cross-linked complex was tryptically digested and cross-linked peptides were purified by size-exclusion chromatography prior to LC-MS/MS analysis on an UPLC (EASY-nLC 1000, Thermo Scientific) online coupled to an LTQ Orbitrap Elite system (Thermo Scientific) as described earlier (Herzog *et al*, 2012). Mass spectrometric data were analyzed using the xQUEST/xPROPHET software suite and further manually validated (Walzthoeni *et al*, 2012). A full list of the cross-links and more details on the CXMS analysis are provided in the source data for Fig 3 and Appendix Supplementary Methods, respectively.

Fluorescence anisotropy measurements

Mre11^{RBD}–Rad50^{NBD} dilutions were prepared in assay buffer, incubated for 1 h, and then mixed with labeled dsDNA (at a final concentration of 50 nM) in a 1:1 (v/v) ratio. After equilibration, the fluorescence anisotropy was measured. The data were analyzed with Prism (Version 6 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.) and fit to a Hill model as well as a single-site binding model accounting for receptor depletion (Figs 6 and EV4; Appendix Table S2). For details, see Appendix.

Plate survival assay

The plate survival assay with *S. cerevisiae* carrying wild-type or mutated Rad50 allele was performed as described before (Rojowska *et al*, 2014). Briefly, freshly growing cells from a plate were resuspended in deionized water and diluted to OD₆₀₀ of 1 and serial

tenfold dilutions were prepared. W303-1a wild-type and W303-1a $\Delta rad50$ strains were kind gifts from Katja Strässer and Steve Jackson, respectively.

Accession codes

Coordinates and structure factors have been deposited in the Protein Data Bank under accession codes 5DA9 for CtMre11^{RBD}–Rad50^{NBD} and 5DAC for CtRad50^{RBD}–DNA.

Expanded View for this article is available online.

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Author contributions

FUS, KL, and KPH designed the experiments. FUS cloned, purified, and crystallized the proteins with help of BK, FUS and KL analyzed SAXS and X-ray data and FUS built the atomic models. GS performed and evaluated the CXMS experiment and FUS carried out fluorescence anisotropy measurements. BK, FUS, and KL performed the yeast assays. KPH supervised the research.

Conflict of interest

The authors declare that they have no conflict of interest.

Note added in proof

While this paper was under final review, Cho and colleagues published the structure of the *Methanocaldococcus jannaschii* Mre11–Rad50–ATPγS–DNA complex, revealing how archaeal Rad50–Mre11 interacts with DNA in an ATP-dependent fashion (DOI: 10.15252/embj.201592462).

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