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The Mre11/Rad50/Nbs1 Complex: recent insights into catalytic activities and ATP-driven conformational changes

Tanya T. Paull[*] and **Rajashree A. Deshpande**
The Howard Hughes Medical Institute, The Department of Molecular Biosciences, and the Institute for Cellular and Molecular Biology, The University of Texas at Austin, Austin, TX 78712, USA

Abstract

DNA double-strand breaks (DSBs) can arise from internal or external sources of damage, and the rapid detection, processing, and repair of this damage is important for cell viability. Failure to repair DNA damage can result in genomic instability, ultimately increasing the frequency of lymphoid disorders, neurodegeneration, and cancer. The Mre11-Rad50-Nbs1 (Xrs2) complex plays a central and critical role in detection and repair of DSBs and is conserved in all kingdoms of life, as Mre11/Rad50 (MR) in prokaryotes and as MRN/X in eukaryotes (Lamarche et al., 2010; Stracker and Petrini, 2011). The importance of this complex is emphasized by the fact that deletion of any of the three components results in embryonic lethality in mice and loss of proliferative activity in embryonic stem cells (Buis et al., 2008; Luo et al., 1999; Xiao and Weaver, 1997; Zhu et al., 2001) which is likely related to the role of MRN/X in homologous recombination. Repair of DSBs by homologous recombination involves replication of the broken region using an undamaged template, usually a sister chromatid. Deletions of other genes important for homologous forms of repair also exhibit early embryonic lethality, including Rad51, BRCA1, BRCA2, and CtBP-interacting protein (CtIP)(Chen et al., 2005b; Gowen et al., 1996; Lim and Hasty, 1996; Sharan et al., 1997). Hypomorphic mutations in MRN components result into developmental and neurodegenerative disorders in humans, including Ataxia-Telangiectasia-Like Disorder (ATLD), Nijmegen Breakage Syndrome (NBS), and NBS-like syndrome (Matsumoto et al., 2011; Stewart et al., 1999; Varon et al., 1998; Waltes et al., 2009), which are related, at least in part, to the role of MRN/X in the activation of cell-cycle checkpoints through the Ataxia-Telangiectasia-Mutated (ATM) protein kinase (Lee and Paull, 2007; Shiloh and Ziv, 2013). The roles of MRN/X also extend to the processing of DSBs during meiosis, for which it is essential, and to telomere maintenance (Borde, 2007; Lamarche et al., 2010).

[*]corresponding author: tpaull@utexas.edu.

Repair of DSBs is achieved through two broadly-defined groups of pathways: nonhomologous end joining (NHEJ) and homologous recombination (HR) (Krogh and Symington, 2004). The choice between these pathways primarily depends on the cell-cycle phase and the complexity of the damage generated at the break site (Chapman et al., 2012; Schipler and Iliakis, 2013). In the classical NHEJ pathway, ends are bound by the Ku70–Ku80 heterodimer/DNA-dependent protein kinase catalytic subunit (DNA-PKcs) complex which recruits additional factors involved in end modifications and gap filling. DNA ends are ultimately ligated by the NHEJ-specific DNA ligase IV complex (Deriano and Roth, 2013). In mammalian cells, the C-NHEJ pathway is not dependent on the MRN complex, although in budding yeast MRX contributes to NHEJ pathway through interactions with Ku70-Ku80 and DNA Lig4 complexes (Lewis and Resnick, 2000). The MRN complex, in conjunction with CtIP/Sae2, also regulates the alternative NHEJ (A-NHEJ or MMEJ), which utilizes short microhomologies and can result in large deletions (Lee and Lee, 2007; Yun and Hiom, 2009). In mammalian cells MRN was also shown to interact with DNA ligaseIIIα/Xrcc1, the ligase complex implicated in alternative NHEJ, stimulating intermolecular ligation (Della-Maria et al., 2011).

In contrast to NHEJ, HR requires the 5′–3′ resection of dsDNA to generate single-stranded DNA tails, a process that is initiated by the MRN complex and CtIP (You and Bailis, 2010). Extensive resection is perfomed by exonuclease 1 (Exo1), and Dna2 (Symington and Gautier, 2011), whose activities are also promoted by MRN (Cejka et al., 2010; Nicolette et al., 2010; Niu et al., 2010; Yang et al., 2013; Zhou et al., 2014; Zhou and Paull, 2013). 3′ ssDNA tails thus generated are bound by replication protein A (RPA), which activates ATM- and Rad3-Related (ATR), promoting replication checkpoint arrest and stabilization of replication forks (Zeman and Cimprich, 2014). RPA on these 3′ ssDNA tails is then exchanged for Rad51 to create Rad51 filaments that catalyze homology search and strand invasion, ultimately priming DNA synthesis and resolution of repair intermediates.

The MRN complex plays important and diverse roles in DNA double-strand break repair and signaling. Here we review recent evidence elucidating the structures and regulation of the Mre11/Rad50 complex, focusing primarily on the enzymatic activities of MRN and the role of ATP-driven conformational changes in Rad50.

Mre11 nuclease activity

The Mre11 protein is related to a family of phosphoesterases that includes lambda phosphatase, protein phosphatase-2B, PP2A, PP1, calcineurin, and purple acid phosphatases (Koonin, 1994)(Fig. 1). This family of enzymes binds two metal ions in the active site and cleaves either phosphomonoester or phosphodiester bonds. Mre11 is conserved in all species and exhibits manganese-dependent 3′ to 5′ exonuclease and endonuclease activities on double-stranded DNA in vitro (Connelly et al., 1999; Hopkins and Paull, 2008; Paull and Gellert, 1998; Trujillo and Sung, 2001; Trujillo et al., 1998). The roles of these activities in cells have been widely debated and it is still not entirely clear what the biologically relevant activity is, but it is likely that this depends on the structure and context of the DNA ends. Experiments in budding yeast have shown that the nuclease activity of Mre11 is dispensable for the resection of enzymatically-generated DSBs but is absolutely required for meiosis

when DSBs are covalently bound by the Spo11 protein, as well as for the processing of cruciform structures in vegetatively growing cells (Lobachev et al., 2002; Moreau et al., 1999; Rattray et al., 2001). Mre11 nuclease activity also contributes to (but is not essential for) the survival of radiation damage and topoisomerase conjugates in budding yeast (Moreau et al., 1999), although at least some of its activities are redundant with Dna2 (Budd and Campbell, 2009). In vitro experiments show that a nuclease-deficient MRN complex can promote Exo1-mediated resection in the presence of Ku and DNA-PKcs equivalently to that of a wild-type complex (Yang et al., 2013; Zhou and Paull, 2013), further suggesting that Mre11 nuclease activity is not essential for resection of enzymatically generated DSBs. Similarly, the nuclease activity of MRN not necessary for compatible end ligation by DNA ligaseIIIα/Xrcc1, but is critical for the ligation of incompatible DNA ends that require processing (Della-Maria et al., 2011).

The role of Mre11 3′ to 5′ exonuclease activity has been unclear, in part because it requires millimolar levels of manganese in vitro (higher than would ever be encountered in vivo) and because the polarity of the exonuclease is opposite to the 5′ to 3′ resection required for creation of long 3′ ssDNA tails that are ultimately bound by Rad51. Recent support for a physiological role of the exonuclease activity came from a study of meiotic DSB repair in which breaks are created by and covalently linked to Spo11 on the 5′ strands (Garcia et al., 2011). Although Mre11 nuclease activity was known to be required for removal of Spo11, Neale and colleagues showed in this work that the 3′ to 5′ exonucleolytic activity of Mre11 is specifically required to resect the Spo11-linked strand after an endonucleolytic break is first made, approximately 300 nt from the Spo11 cut; 5′ to 3′ exonucleolytic degradation from the nick was found to be dependent on Exo1 (Fig. 2).

A similar model was proposed for HR in mammalian cells, where the roles of Mre11 exo- and endonuclease activites were assessed through the use of small molecule inhibitors that specifically affect each of these activities (Shibata et al., 2014). It was suggested that Mre11 endonucleolytic activity initiates resection, followed by Mre11-dependent 3′ to 5′ exonuclease and Exo1/BLM-dependent bidirectional resection from the site of the nick. The catalytic functions of Mre11 appear to be much more important in mammals than they are in yeast, as a transgenic mouse model expressing nuclease-deficient Mre11 exhibited early embryonic lethality (Buis et al., 2008). In further support of a physiological role of Mre11 exonucleolytic activity in mammalian cells, Mre11 was found to be responsible for the degradation of nascent strands at stalled replication forks in the absence of BRCA2 (Schlacher et al., 2011). Mre11 endonucleolytic activity in manganese has been observed in vitro with all Mre11 orthologs studied (Connelly et al., 1998; Herdendorf et al., 2011; Paull and Gellert, 1999; Trujillo and Sung, 2001). A weak but detectable endonuclease activity was also observed on 5′ strands of DSBs in the presence of magnesium using *P. furiosus* MR (PfMR)(Hopkins and Paull, 2008). The oligonucleotide products of this activity were found to be 10 to 50 nt long and were eliminated by mutations that inactivate the exonuclease activity, confirming that the activity resides in the same active site. pfMR was shown in this work to process DSB ends cooperatively with the helicase nuclease complex HerA/NurA that is expressed from the same operon in *P. furiosus* and other archaea species (Constantinesco et al., 2004). Magnesium-dependent nuclease activity has also been

observed with gp46/47 (phage T4 Mre11/Rad50) in vitro (Herdendorf et al., 2011). Interestingly, with T4 MR, other proteins were found to promote the endonucleolytic activity of Mre11 in magnesium, including the recombination mediator protein UvsY and the ssDNA binding protein gp32 (Herdendorf et al., 2011).

It is still an open question what metal ions exist in Mre11 proteins in vivo, and thus what nuclease activities the proteins possess under physiological conditions. We recently performed partial proteolysis experiments with human Mre11 in vitro and found that oxidative cleavage of the protein occurred in the presence of ascorbic acid and hydrogen peroxide but without any added metal (Makharashvili et al., 2014). We furthermore mapped the sites of cleavage within Mre11 to amino acids 131 and 217, both residues directly contacting metal ion 2 in the structure of human Mre11 (Park et al., 2011). This evidence strongly suggests that there is a stably-bound transition metal ion bound to Mre11 in site 2, and is similar to previous experiments showing a high affinity manganese binding site in lambda phosphatase at metal position 2 (White et al., 2001). It will be informative to identify what metals are bound to sites 1 and 2 and to determine the functional consequences of these different states.

Rad50 ATP binding

Rad50 contains Walker A and Walker B ATP-binding motifs and is similar in domain configuration to the Structural Maintenance of Chromosomes family of ATPases that regulate the activities and topology of chromosomal DNA (Williams et al., 2007)(Fig. 1). Two Rad50 catalytic domains come together to form the ATP-bound structure, with the coiled-coils of each protein extending away from the globular domains (Fig. 3). The ATP-related activities of the Rad50 protein are essential for all of its roles in DNA repair and signaling, as mutations in the Walker A or signature motifs exhibit phenotypes equivalent to a *rad50* deletion in vivo (Alani et al., 1990; Bhaskara et al., 2007; Chen et al., 2005a; Moncalian et al., 2004). Even though Rad50 ATPase activity has long been known to be essential for MRN function, the exact role of this activity has not been very clear. In recent years, significant advances were made in understanding the structure of the Rad50 catalytic domain in the absence and presence of ATP, which showed that the protein undergoes a dramatic conformational change between these states (Lammens et al., 2011; Lim et al., 2011; Mockel et al., 2011; Williams et al., 2011). The catalytic head of Mre11-Rad50 complexes is composed of Mre11 nuclease domains and Rad50 ATPase domains. A crystal structure of the catalytic domains of bacterial MR in the absence of ATP shows that Mre11 holds the Rad50 ATPase domains near the base of the coiled coils (Lammens et al., 2011) (Fig. 3). In this configuration, the ATPase domains are separated and facing away from each other, forming an “open” structure. In contrast, the ATP-bound form of MR shows the ATPase domains together, where two ATP molecules are bound within the Rad50 dimer, occluding the Mre11 nuclease active sites and forming a “closed” conformation (Lim et al., 2011; Mockel et al., 2011). A large cavity within the Rad50 domain in the unbound, “open” configuration is important for the large conformational change to the “closed” state and mutations made in this cavity have effects on the relative stability of each state (Deshpande et al., 2014).

The importance of the ATP-bound "closed state"

We have known for many years that the ATPase activity of Rad50 is quite slow. Estimates of the ATPase activity of Rad50 from bacteria, yeast, and humans have yielded rates of 0.03 to 14 moles ATP hydrolyzed per mole of protein per minute, with the eukaryotic complexes generally showing much slower rates of hydrolysis (0.03 to 0.1) (Bhaskara et al., 2007; de Jager et al., 2002) compared to the phage, bacterial, and archaea complexes for which most measurements yield estimates of 1 to 4 moles per minute (Deshpande et al., 2014; Herdendorf et al., 2011; Lammens et al., 2011; Majka et al., 2012). These observations suggest that the complex may occupy the closed state for relatively long periods of time in vivo. Recent evidence shows that many important functions of Rad50 take place with the complex in the ATP-bound but unhydrolyzed state, however, perhaps explaining this long occupancy. We showed several years ago that stable binding of DNA fragments by human MRN is supported by non-hydrolyzable analogs of ATP (Lee et al 2003), and recent evidence suggests that ATP binding but not hydrolysis also promotes DNA end tethering by the pfMR complex (Deshpande et al, 2014). Using single-molecule FRET, ATP-bound human MRN was shown to bind to DSBs in a manner that stably unwinds 15-20 base pairs at the end of duplex, holding the branched structure open for minutes at a time at room temperature (Cannon et al., 2013). This unwound form of DNA present in the closed conformation of Rad50 was predicted from earlier ensemble experiments (Lee and Paull, 2005; Paull and Gellert, 1999) and is required for ATM activation as well as DSB resection in human cells (Cannon et al., 2013; Lee et al., 2013). It is still unclear what stimulates ATP hydrolysis by Rad50, although DNA as well as other DNA-binding proteins were reported to have a marked effect on the ATPase activity of T4 Rad50 (Herdendorf et al., 2011).

Release from the closed state with ATP hydrolysis

An important aspect of the Rad50 closed state is the fact that the Mre11 nuclease active sites are completely occluded by the Rad50 catalytic domains in this conformation (Lim et al., 2011; Mockel et al., 2011). Consistent with the structural predictions, we have found that stabilization of the closed state (either by mutation or by crosslinking) results in loss of Mre11 nuclease activity (Deshpande et al., 2014). These observations are also consistent with early data from analysis of E. coli SbcCD (E. coli MR) and pfMR showing that ATP hydrolysis is essential for the exonuclease activity of Mre11 when bound to Rad50 (Connelly et al., 1997; Hopfner et al., 2000). More recent studies with T4 MR have provided further detail for this model, showing that ATP hydrolysis is required for repetitive nucleotide removal but not required for removal of first nucleotide by nuclease activity, suggesting that ATP hydrolysis is likely involved in translocation of the complex (Herdendorf et al., 2011). Human Mre11 can act independently from Rad50 as a 3′ to 5′ exonuclease (Paull and Gellert, 1998), unlike the prokaryotic enzymes, again confirming that Rad50 ATP hydrolysis is not essential for Mre11 nuclease activity per se but serves to restrain and regulate the nuclease functions of Mre11.

Although a structure of PfMre11 bound to two DNA molecules has been published in which the Mre11 dimer interface adopts at least two different states during the exonuclease reaction (Williams et al., 2008), it is not yet clear how the closed state of MR transitions to a

conformation in which the ends of DNA are accessible to the Mre11 active site. We envision that an intermediate state occurs after ATP hydrolysis that positions the Mre11 active sites such that they form productive complexes with DNA (Fig. 3). Understanding these details will likely require structural analysis of MR intermediates with DNA ends.

The coiled-coil domains of the Rad50 protein

Rad50 has structural features similar to SMC proteins and harbors a long coiled-coil that coordinates a Zn atom with its tip (Fig. 1). There is some variation in the length of the coiled-coil region across complexes from different species (de Jager et al., 2004). As observed with AFM, the average contour length of the complex varies from 39 nm observed with the *E. coli* SbcC/D MR complex (660 amino acids) to 47 nm (960 amino acids) in human MR. By primary sequence comparison the coiled-coils of T4 Rad50 are among the shortest, consisting of only 330 amino acids. AFM and EM imaging of the complexes shows that the arms appear in various conformations from completely splayed to closed and the coils can be either straight, bent, or kinked (de Jager et al., 2004; Hopfner et al., 2002). Time resolved analysis of single molecules of MR showed joining and separation of the arms at the tip, as well as changes in curvature indicating these arms to be highly flexible (Moreno-Herrero et al., 2005). The coiled-coil domains clearly respond to ATP binding and hydrolysis through large changes in their conformation, as visualized by crystal structures and AFM. A large swivel of Rad50 coiled coil domains accompanies ATP binding, changing the angle between the coiled-coils domains from 120 to 90 degrees (Figure 3) (Deshpande et al., 2014; Lammens et al., 2011; Lim et al., 2011; Williams et al., 2011).

The mechanistic function of the coils has been elusive, although it is clear that they are essential for Rad50 function in vivo. Ablation of Zn chelation in yeast Rad50 by mutation of the Cys residues in the Zn hook region leads to an increase in IR sensitivity similar to that of a null strain (Hopfner et al., 2002). Complete deletion of the Zn hook abolishes telomere maintenance and meiotic DSB formation, and severely impairs HR as well as NHEJ (Hohl et al., 2011). Similar results were seen for coiled coil truncation mutants, stressing the importance of the length of Rad50 coiled coils, although shortening the coiled coil region by 243 amino acids (retaining the zinc hook) was substantially tolerated for homologous recombination. Using live cell imaging, the Rad50 zinc hook was shown to be important for human MRN localization to the sites of DSBs and for the DNA damage response including homologous recombination and both ATM and ATR activation pathways (He et al., 2012). In addition, cohesin enrichment at replication forks depends on Rad50, and the zinc hook and coiled coils are important for this loading (Tittel-Elmer et al., 2009).

Experiments in vitro have also established the critical role of the coiled-coil domains. Removal of the coils impairs the DNA binding ability of MRN as well as DNA end tethering and ATPase activity (Deshpande et al., 2014; Lee et al., 2013). In addition, loss of the Zn hook-mediated connection between Rad50 molecules results in loss of Nbs1 binding and ATM activation (Lee et al., 2013). Why is the coiled-coil region so important for activities that take place in the globular domains of Rad50 and Mre11? From our in vitro experiments it has become clear that one of the essential roles of the coils and hook structure is to physically link the Rad50 catalytic heads together. In the absence of the coils/hook,

Rad50 catalytic heads do not stably form a complex together with ATP (Deshpande et al., 2014). This makes intuitive sense because the Rad50 catalytic heads need to have a low affinity for each other in order to release from the closed state with ATP hydrolysis. The presence of the zinc hook connection increases the local concentration of the Rad50 catalytic heads such that the association between the heads is strongly favored and can even occur in the absence of ATP (Deshpande et al., 2014).

More difficult to answer is the question: why are the Rad50 coiled-coils so long? Results from coil deletion experiments in which shortened coils have been connected together either by the zinc hook itself or a heterologous linkage show that the proper length of the coils is essential for normal function (Hohl et al., 2011). One possibility that is often depicted in diagrams of MRN is that the coils physically connect two Rad50 molecules that are each bound to different DNA ends; however, it is difficult to determine this from the resolution of AFM images. MR proteins have been visualized tethering multiple linear DNA molecules into large, protein-bound complex, perhaps with the coiled-coil domains mediating these interactions (de Jager et al., 2001). These observations are consistent with the known DNA tethering activity of the complex and would allow for flexible, multipartite interactions between DNA ends. Another theoretical possibility is that the Rad50 coils actually encircle multiple DNA molecules, similar to cohesins, although there is currently no data supporting such a role.

Roles of Nbs1 in Regulation of MR

The Nbs1/Xrs2 component of MRN in eukaryotic cells does not possess enzymatic activity, but regulates the activities of Mre11 and Rad50 (Paull and Gellert, 1999) and is responsible for localizing MR to the nucleus in mammalian cells (Desai-Mehta et al., 2001). Recent structures of Mre11 from humans and fission yeast revealed parts of the Nbs1 interaction region on Mre11 (Park et al., 2011; Schiller et al., 2012), showing that two Nbs1 subunits stretch around the outside of nuclease domains of Mre11. We do not have structural information about the entire MRN catalytic head domain complex, but it is clear from in vitro biochemistry that Nbs1 stabilizes the ATP-bound form of MR and appears to be required for the ATP-dependent functions of the complex (Lee et al., 2003; Lee et al., 2013; Paull and Gellert, 1999). Nbs1 also contains an ATM-binding region at its C-terminus that is critical for ATM activation via MRN and DNA DSBs (Falck et al., 2005). It is not clear what the mechanistic role of this peptide is in activating ATM but in vitro studies with recombinant ATM also show this requirement for the Nbs1 C-terminus (Lee et al., 2010). Interestingly, we also found in this study that the mediator protein 53BP1 can compensate for the loss of the Nbs1 C-terminus in vitro in promoting ATM activity, most likely due to its ability to bind to both ATM and MRN. MR can also bind to ATM independently of Nbs1 (Lee and Paull, 2004) through a binding site in the Rad50 protein (JH Lee and T.Paull, unpublished observations).

The MRN complex associates with DSB sites through at least two different mechanisms. Binding to the chromatin domain containing the break requires phosphorylation of the histone variant H2AX by ATM and DNA-PKcs, which is subsequently bound by the mediator protein Mdc1 (Stucki et al., 2005). Nbs1 contains N-terminal FHA and BRCT

domains that bind to Mdc1 at constitutively phosphorylated CK2 sites, localizing MRN to sites of DNA damage (Chapman and Jackson, 2008; Melander et al., 2008; Spycher et al., 2008). MRN also is thought to localize directly to break sites in a much smaller region of chromatin visible by immunofluorescence, independent of Mdc1 (Lukas et al., 2004); this localization may be dependent at least in part on the replication checkpoint protein Rad17 (Wang et al., 2014). The N-terminal FHA domain of Nbs1 in fission yeast also associates with the DSB repair factor Ctp1, the ortholog of CtIP, which is required for the efficient repair of breaks in *S. pombe* (Dodson et al., 2010; Williams et al., 2009). Direct binding between human MRN and CtIP has been well-documented (Chen et al., 2008; Sartori et al., 2007; Yuan and Chen, 2009).

Overall, the MRN complex is a multifunctional enzyme assembly with several distinct activities in the DNA damage response. Mre11 nuclease activity is tightly regulated by the binding and ATP hydrolysis of the Rad50 protein, and these two enzymatic components are regulated in turn by Nbs1. The association of MR and Nbs1 with ATM and the regulation of ATM by MRN serve to link this central DNA end recognition complex with the primary signaling kinase in eukaryotic cells to coordinate DSB repair with checkpoint activation. There are many aspects of this relationship that we still do not understand but the recent and ongoing structural and biochemical insights into the complex bode well for this goal being achieved.

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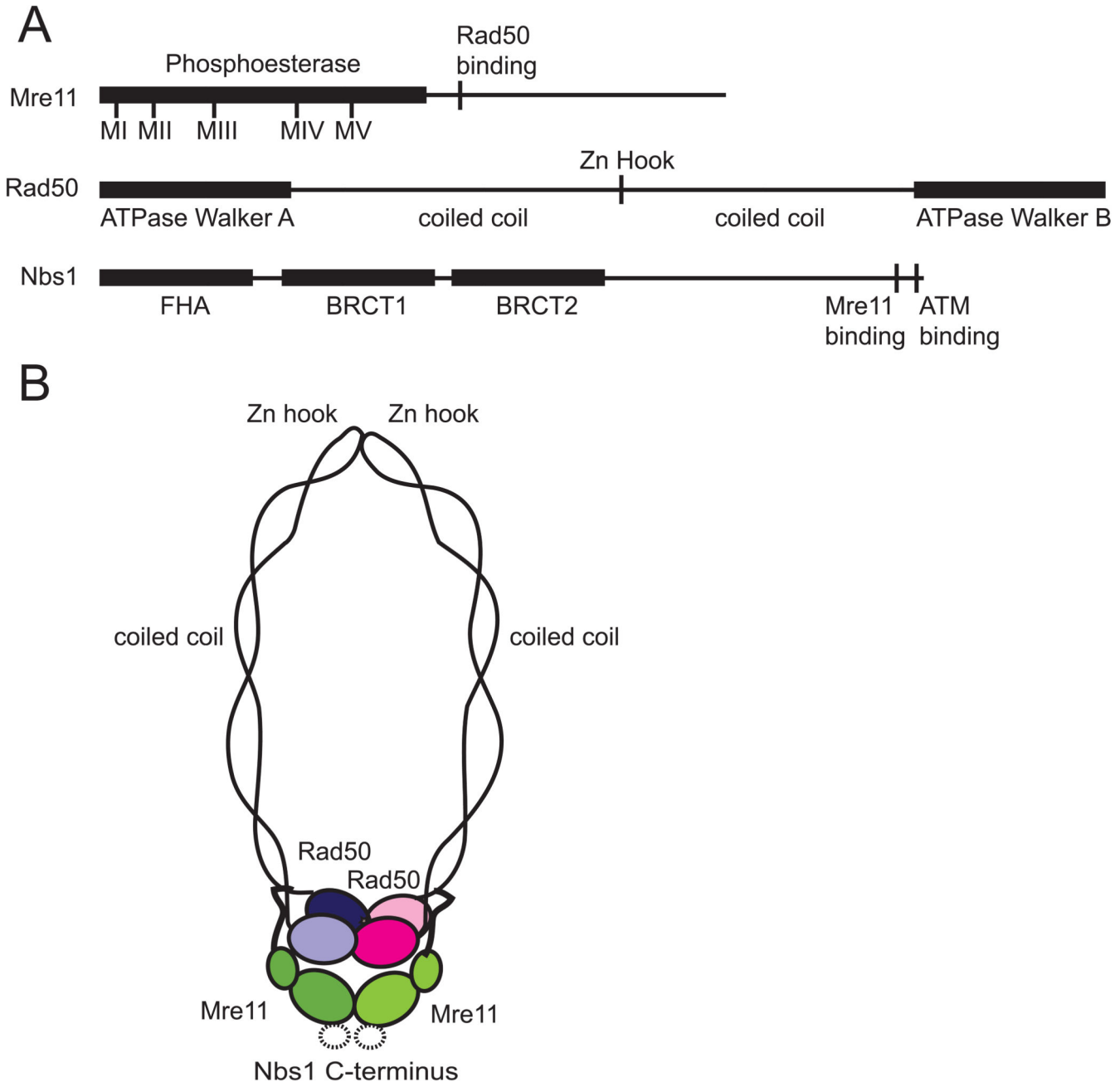

Figure 1.

Components of the MRN complex. (A) Linear maps of Mre11, Rad50 and Nbs1 showing functional domains, including the conserved phosphoesterase motifs in Mre11, Walker A and B ATP-binding motifs in Rad50 and the FHA and BRCT domains in Nbs1. (B) Interactions among the components of the MRN dimer. The globular domain is comprised of Mre11 phosphoesterase/nuclease domains, Rad50 ATPase domains, and Nbs1. The two Rad50 molecules also interact with a Zn hook at the tip of coiled coils. Binding site for Nbs1 is based on crystallographic data (Park et al., 2011; Schiller et al., 2012).

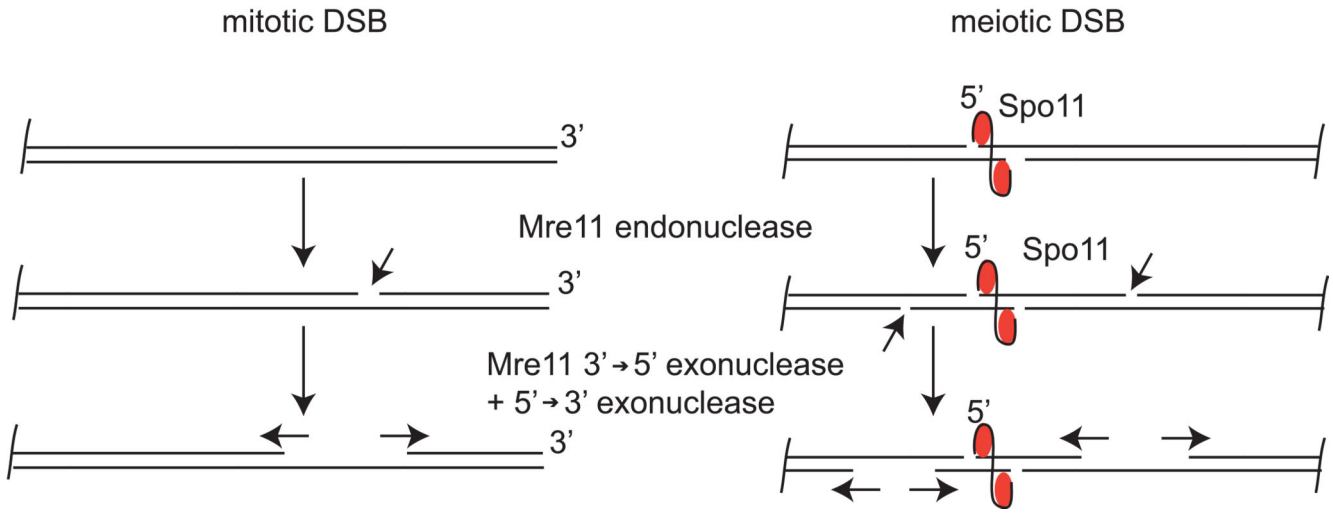

Figure 2.

Model for the role of Mre11 catalytic activities during repair of DSBs in mitotic homology directed repair (left) and meiotic recombination (right) based on studies in budding yeast and mammalian cells (Garcia et al., 2011; Shibata et al., 2014). An Mre11-dependent endonucleolytic cut is made at a distance from DSB. Mre11 exonuclease activity is proposed to process the single-strand break in the 3′ to 5′ direction towards the DSB and other nucleases (for instance Exo1) would continue resection in the 5′-3′ direction away from the DSB.

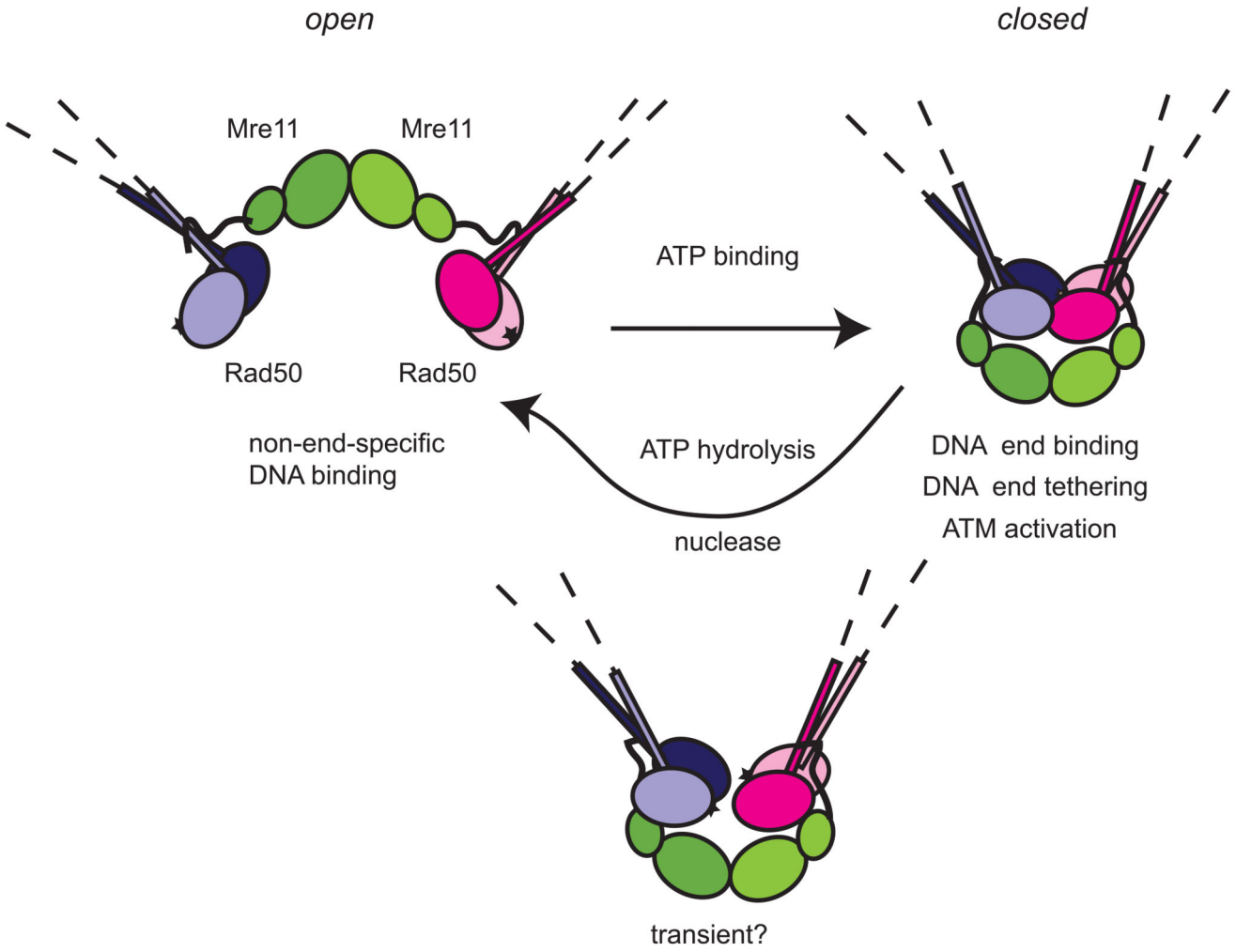

Figure 3.

ATP induced conformational changes in the catalytic head of MRN complex. The globular domain comprised of Rad50 ATPase and Mre11 nuclease domains is depicted as revealed from crystal structures (Lammens et al., 2011; Lim et al., 2011; Mockel et al., 2011). Mre11 binds Rad50 at the base of coiled coils. In the ATP unbound form, the structure is “open”, with Mre11 nuclease active sites accessible. ATP binding sites are shown as stars. In this state, the complex can engage DNA in a non-end specific manner (Deshpande et al., 2014). Binding of ATP brings the ATPase domains together forming a “closed” state. This form promotes end specific DNA binding and DNA tethering by MR/MRN complex and ATM checkpoint activation (Lee et al., 2013). Although this form blocks the nuclease site, ATP hydrolysis followed by separation of the ATPase domains is required for nuclease activity of Mre11, likely through a transient intermediate, although the structure of this theoretical conformation is unknown.