Mre11 ATLD17/18 mutation retains Tel1/ATM activity but blocks DNA double-strand break repair

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Received August 6, 2012; Revised September 19, 2012; Accepted September 20, 2012

ABSTRACT

The Mre11 complex (Mre11-Rad50-Nbs1 or MRN) binds double-strand breaks where it interacts with CtIP/Ctp1/Sae2 and ATM/Tel1 to preserve genome stability through its functions in homology-directed repair, checkpoint signaling and telomere maintenance. Here, we combine biochemical, structural and in vivo functional studies to uncover key properties of Mre11-W243R, a mutation identified in two pediatric cancer patients with enhanced ataxia telangiectasia-like disorder. Purified human Mre11-W243R retains nuclease and DNA binding activities in vitro. X-ray crystallography of Pyrococcus furiosus Mre11 indicates that an analogous mutation leaves the overall Mre11 three-dimensional structure and nuclease sites intact but disorders surface loops expected to regulate DNA and Rad50 interactions. The equivalent W248R allele in fission yeast allows Mre11 to form an MRN complex that efficiently binds double-strand breaks, activates Tel1/ATM and maintains telomeres; yet, it causes hypersensitivity to ionizing radiation and collapsed replication forks, increased Rad52 foci, defective Chk1 signaling and meiotic failure. W248R differs from other ataxia telangiectasia mutated kinase via Nbs1. ATM stimulates repair factors through phosphorylation and initiates the checkpoint response that stalls cell cycle progression during DNA repair, or activates senescence or apoptosis pathways. Nbs1 also recruits the DNA end-processing factor CtIP/Ctp1/Sae2, which along with the nuclease activity of Mre11, initiates the 5' to 3' resection of DNA ends that is essential for homologous recombination (HR) repair of DSBs (4–8). Extended DNA end resection is catalyzed by Exo1 exonuclease, or an activity dependent on a RecQ-family DNA helicase, Dna2 nuclease and Rmi1-Top3 topoisomerase, to generate 3' single-stranded DNA (ssDNA) overhangs (9–13), which perpetuate the checkpoint response via the ATRRad3-ATRIPRad26 complex binding to RPA-coated ssDNA, and facilitate homology-directed repair mediated by Rad51 (14).

INTRODUCTION

DNA double-strand breaks (DSBs) are a driving force in genome instability, as they are a primary source of chromosome breaks that can lead to chromosome translocations or loss of chromosome fragments (1). The MRN complex, composed of Mre11, Rad50 and Nbs1 (Xrs2 in Saccharomyces cerevisiae) subunits, has crucial roles in genomic stability through its functions in DNA repair, checkpoint signaling and telomere maintenance (2). As a primary responder to DNA DSBs, the MRN complex rapidly localizes to damage sites and recruits the ATM/Tel1 (Ataxia telangiectasia mutated) kinase via Nbs1. ATM stimulates repair factors through phosphorylation and initiates the checkpoint response that stalls cell cycle progression during DNA repair, or activates senescence or apoptosis pathways (3). Nbs1 also recruits the DNA end-processing factor CtIP/Ctp1/Sae2, which along with the nuclease activity of Mre11, initiates the 5' to 3' resection of DNA ends that is essential for homologous recombination (HR) repair of DSBs (4–8). Extended DNA end resection is catalyzed by Exo1 exonuclease, or an activity dependent on a RecQ-family DNA helicase, Dna2 nuclease and Rmi1-Top3 topoisomerase, to generate 3' single-stranded DNA (ssDNA) overhangs (9–13), which perpetuate the checkpoint response via the ATRRad3-ATRIPRad26 complex binding to RPA-coated ssDNA, and facilitate homology-directed repair mediated by Rad51 (14).
The loss of Mre11 or mutations that ablate its endonuclease activity cause early embryonic lethality in mice and acute clastogen sensitivity in Schizosaccharomyces pombe (8,15). Mre11 is also crucial in S. cerevisiae, although the effects of ablating its nuclease activities are weaker (16,17). Inherited hypomorphic mutations in Mre11 are associated with ataxia telangiectasia-like disorder (ATLD), a rare disease characterized by progressive cerebellar ataxia, an increase in chromosomal aberrations and sensitivity to ionizing radiation (IR) (18–20). ATLD was identified as a milder variant of a condition called ataxia telangiectasia (A-T), which results from mutations in the ATM kinase. A-T patients present with the same clinical features of A-T, which results from mutations in the ATM kinase. Mre11 or ablate its ability to form a complex with Rad50 and Nbs1 that recruits Tel1 to DSBs, but it disturbs interactions amongst the MRN subunits resulting in defects in DNA end processing.

### MATERIALS AND METHODS

#### Protein expression and purification

Expression and purification of P. furiosus Mre11 (residues 1-342) followed published procedures (26). The L204R mutation was introduced by Quickchange Site Directed Mutagenesis Kit (Agilent).

C-terminal tagged 6-histidine human Mre11 preparations were produced by infection of SF9 cells in suspension culture with baculoviruses and collected after 72 h. Proteins were purified using Ni²⁺-NTA agarose beads (Qiagen). Superdex 200 size-exclusion column (GE Healthcare) and Hitrap Q column (GE Healthcare) performed sequentially. Purified Mre11 was pooled and concentrated on a Heparin column (GE Healthcare). Immunoblotting of Mre11 was performed on nitrocellulose membranes (Whatman) using standard immunoblotting techniques. Protein concentrations were determined by comparison with a bovine serum albumin standard on a 10% polyacrylamide gel after staining with Coomassie blue. Detailed procedure is available in Supplementary Data.

#### Crystallization, X-ray diffraction data collection, structure determination and refinement

Mre11 crystals were grown by mixing 1 μl protein solution (20 mg/ml in 200 mM NaCl, 20 mM Tris–HCl pH 7.5, 5% glycerol) and 1 μl crystallization solution (100 mM Bicine, 10% (w/v) PEG6000, 1 M lithium chloride) (ref. cond. 7 JCSG core II suite QIAGEN). Crystals were grown at 15°C by hanging-drop vapor diffusion for 7 days. Crystals were transferred to cryoprotectant (crystallization solution plus 25% PEG200) and flash cooled in liquid nitrogen. Diffraction data were collected and refined to 2.3 Å resolution (Supplementary Table S1). Coordinates for P. furiosus Mre11-L204R crystal structure are available in the Protein Data Bank (PDB: 4HD0).

#### Electrophoretic mobility shift assays

Aliquots of ssDNA90, labeled with Alexa Fluor 488 at the 5′ end, and dsDNA90, labeled with Alexa Fluor 488 at the 5′ end of the upper strand were incubated with the indicated amounts of protein dimer. Substrate sequences and binding buffer composition are available in Supplementary Data. The reaction products were separated on 5% native polyacrylamide gels run in 0.5X TBE at 4°C. Labeled DNA was visualized by direct scanning of the gel with a 488-nm laser using a Typhoon 9200 (GE Healthcare). The emission signal was sorted with a 520BP40-nm filter. Quantification was performed with ImageQuant 5.2 software with error bars representing standard error from four independent experiments.

#### Nuclease assays

Endo- and exonuclease assays were performed with φX174 circular ssDNA virion DNA (New England Biolabs) or linear blunt-ended pBluescript substrates incubated with purified PfMre11 at 50°C or HsMre11 at 37°C for the indicated time points (see Supplementary Data for details). DNA was resolved on tris-acetate-EDTA agarose gels, stained with ethidium bromide and visualized by scanning of gel with a Typhoon 9200.

#### General S. pombe methods

Strains were generated and manipulated using standard techniques (27) and are listed in Supplementary Table
S2. For DNA damage sensitivity assays, 5-fold serial dilutions of exponentially growing cells were spotted on agar plates and treated with the indicated dose of DNA damaging agents. Cultures for Rad52-red fluorescent protein (RFP) foci quantification were grown in minimal media at 25°C with data representative of the mean from quantification of three sets of ~400 cells observed by live-cell fluorescence microscopy (error bars indicate standard deviation from the mean). Spore viability and chromatin immunoprecipitation (ChIP) experiments were performed as described previously (28) with HO endonuclease expression driven by thiamine repressible promoter, mnt41. Samples were collected at the indicated time points after removal of thiamine. Microscopic analysis confirmed cell elongation in ~80% of cells, indicating efficient cutting of the HO site causing a cell cycle arrest before sample collection. Data shown are representative of three independent experiments.

Western blots
Whole-cell extracts were prepared from exponentially growing cells in standard NP-40-based lysis buffer. For MRN blotting, proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) using 3–8% or 7% tris-acetate gels per manufacturer instructions (Life Technologies). Membranes were blotted with PAP (Sigma P1291), MYC (Convance MMS-150P), FLAG (Sigma F3165) and tubulin (Sigma T5168) antibodies. IPs were performed using Protein G or Tosylactivated Dynabeads (Life Technologies) conjugated to the indicated antibody or rabbit IgG (Sigma I5006) with whole-cell extracts representing ~1% of the input. For H2A, cells were harvested 30 min with or without 90 Gy of IR treatment. Total protein was resolved by SDS-PAGE on 4–20% tris-glycine gels (Life Technologies). Blocking and blotting were performed with Odyssey Blocking Buffer (Li-Cor) per manufacturer instructions and incubated with a rabbit polyclonal phospho-specific Histone H2A antibody (29) or total H2A (Active Motif 39235). Blots were incubated with goat anti-rabbit antibody conjugated to an infrared dye (Li-Cor 827-11081) and scanned and quantified with Odyssey Infrared Imaging System (Li-Cor) with an intensity of 827-11081) and scanned and quantified with Odyssey Imaging System at intensity 7. Image shown is representative of three independent experiments.

RESULTS

Mre11-W243R retains in vitro DNA binding and nuclease activities

The two novel mutations in ATLD17/18 patients occur in the Mre11 core, which consists of nuclease and cap domains (25). The g.2499 G>A mutation generates an alternative mRNA splice resulting in the loss of 27 amino acids (Ile340-Arg366). This severe deletion, which removes a large part of the helix zF in the cap domain, is predicted to destabilize the protein (32). The c.727 T>C mutation replaces tryptophan-243 with arginine. This substitution maps just upstream of the well-conserved nuclease motif V in the N-terminus of Mre11 (Figure 1A and B).

To initiate investigations of the Mre11-W243R mutation, we purified human Mre11 for electrophoretic mobility shift assays (EMSSAs) with single-stranded and double-stranded 90-mer DNA substrates (Figure 1C). Mre11-W243R efficiently bound both substrates. In fact, it bound both DNA substrates better than wild-type Mre11 (Figure 1D). To determine whether the mutation affected ssDNA endonuclease activity, Mre11-W243R was incubated with a circular ssDNA plasmid substrate. Mre11-W243R endonuclease activity was unimpaired and appeared hyper-efficient (Figure 1E), which could possibly reflect its higher affinity for ssDNA. In the case of exonuclease assays performed with a linearized dsDNA, wild-type and mutant Mre11 had similar activity levels (Figure 1F). These data indicate that W243R may alter how Mre11 interacts with DNA, but it does not diminish its intrinsic nuclease activities.

Intact nuclease site in X-ray crystal structure of PfMre11-L204R

The W243R mutation does not ablate the in vitro DNA binding or nuclease activities of Mre11, even though the substitution occurs just upstream of the well-conserved nuclease motif V in the N-terminus of Mre11. To provide structural insights into the mutation, we turned to X-ray crystallography of P. furiosus Mre11. We reasoned that although there are differences in the structures of human and P. furiosus Mre11, the assembly of the nuclease site is conserved (26,32,33). Overlays of Mre11 nuclease domains sites confirmed alignments indicating that Trp243 in human Mre11 (blue) and Leu204 in PfMre11 (cyan) occupy analogous positions within the hydrophobic core of the active site (Figure 2A). We determined the X-ray crystal structure of PfMre11-L204R (1-342), comprising the nuclease and cap domains, at 2.3Å resolution (Supplementary Table S1). Superimposition of PfMre11-L204R dimer (green) with wild-type PfMre11 dimer (cyan) reveals that the mutant maintains its overall structure and its ability to dimerize (Figure 2B). The active site core containing the L204R mutant backbone is assembled similarly to wild-type (Figure 2C), and 2Fe–Fe difference electron density map reveals that nuclease-critical residues, such as His52
and His85 required for exonuclease activity and stabilization of the transition state (for both exo- and endonuclease activities), respectively (8), are unaffected by L204R (Figure 2D). Indeed, consistent with our HsMre11-W243R DNA binding and nuclease assays (Figure 1), purified PfMre11-L204R maintained in vitro DNA binding and endonuclease activities (Figure 2E–G).

Interestingly, we observed that L204R causes the misfolding of three loops (red) in one subunit of PfMre11 (Figure 2B and Supplementary Figure S1), likely due to the torsional stress induced by the charged substitution. These loops are implicated in DNA recognition and binding by in silico DNA docking studies (Supplementary Figure S2) and by the different migration of DNA–protein complexes in EMSAs compared with wild-type Mre11 (Figure 2E). Interestingly, loop L3 maps to the same helix 2F of the cap domain that is truncated in the del(1340-R366) allele of ATLD17/18 (Supplementary Figure S2) that also forms an interface with Rad50 (34,35). Collectively, these data suggest that neither HsMre11-W243R nor PfMre11-L204R disrupt the architecture of the Mre11 nuclease site or its intrinsic nuclease activities, but are more likely to alter how the MRN complex binds DNA and interacts with Rad50 and possibly Nbs1.

*S. pombe mre11-W248R* mutant is hypersensitive to DNA damage

To characterize Mre11-W243R in vivo, we introduced the analogous mutation into a 13MYC-tagged genomic copy
of the fission yeast *S. pombe* Mre11 (Rad32). The un-mutated *mre11*+13MYC strain was as resistant to DNA damage as wild-type, suggesting that the MYC tag did not alter Mre11 function (Figure 3A). The *mre11*-N122S and -W215C alleles, corresponding to human N117S and W210C from ATLD3/4 and ATLD7/8 respectively, were largely insensitive to DNA damaging agents. These results confirm previous studies (36). In contrast, *mre11*-W248R cells displayed acute clastogen sensitivity, approaching the level of *mre11*Δ (Figure 3A). As seen with *mre11*Δ, the W248R mutant formed smaller colonies that consisted of many elongated cells (Supplementary Figure S3), which indicates a defect in repairing spontaneous DNA damage, leading to activation of the checkpoint that delays the onset of mitosis. Interestingly, the *mre11*-W248R mutant was even more sensitive to DNA damage than the *mre11*-H134S nuclease-dead mutant (8).

**Increased Rad52 foci in *mre11*-W248R mutant**

To confirm that *mre11*-W248R cells were elongated owing to the presence of DNA damage, we analysed the formation of Rad52 (Rad22) foci in exponentially growing cells.
Figure 3. Analysis of Mre11 ATLD site mutant in *S. pombe*. (A) 5-fold serial dilutions of Mre11 ATLD and nuclease dead (H134S) mutants were spotted and treated with indicated dose of genotoxic agents. The MYC-tagged Mre11 behaved similarly to the wild-type strain, indicating that the fusion does not alter protein function. IR = ionizing radiation, CPT = camptothecin, UV = ultraviolet radiation, HU = hydroxyurea, MMS = methyl methanesulfonate. (B) Percentage of cells with one or more spontaneously occurring Rad52-RFP focus. At least 1200 cells were analysed for each strain with error bars representing standard deviation from the mean of three experiments. (C) Schematic of chromosome I containing HO break site used in ChIP experiments indicating relative distances and expected sizes assayed by multiplex PCR. (D) ChIP of untagged, Mre11-MYC and Mre11-W248R-MYC samples collected at indicated time points after removal of thiamine from the media, leading to HO endonuclease induction. Small increases in ChIP signals are present around the HO site in the untagged controls. (E) Expression levels of endogenous MRN subunits in Mre11 mutant backgrounds. Tubulin is a loading control.
Rad52 forms bright foci while mediating assembly of Rad51 (Rhp51) onto ssDNA during HR repair (37,38). Approximately 35% of mre11Δ cells had spontaneous Rad52-RFP foci, compared with only 2% in wild-type (Figure 3B). The mre11-W248R and mre11-H134S cells also had elevated levels of Rad52 foci at 22% and 29%, respectively. These results indicate increased genome instability in mre11-W248R and mre11-H134S cells from elevated levels of spontaneously occurring DNA damage and/or defects in its repair. These effects contrast with mre11-N122S and mre11-W215C, which did not differ from wild-type.

**Increased retention of Mre11-W248R at a DSB**

To address whether Mre11-W248R binds DSBs in vivo, we performed ChIP at a site-specific DSB generated by HO endonuclease. Multiplex PCR detected a strong Mre11-W248R signal directly adjacent (0.2 kb) to the break site, in comparison with the modest enrichment of wild-type Mre11 (Figure 3C and D). Inactivation of Mre11 nucleasome activities or elimination of Ctp1 increases the ChIP signal of MRN subunits at the HO DSB, suggesting that DNA end processing by the MRN-Ctp1 complex is required for its departure from the DSB (5,30). Thus, the increased enrichment of Mre11-W248R at HO DSB indicates a defect in DNA end processing by MRN complex in vivo.

Immunoblotting indicated that Mre11-W248R abundance was reduced ~50% relative to wild-type or other ATLD alleles (Figure 3E). This reduction may enhance the mre11-W248R phenotype; however, it is unlikely to be its primary cause. All MRN subunits are haplosufficient for meiosis, which places a heavy burden on the DSB repair machinery. Also, all Mre11-ATLD alleles reduce the abundance of Nbs1, including those that cause little or no phenotype (Figure 3A and E). Indeed, the hyper-enrichment of Mre11-W248R at the HO DSB suggests that its abundance is not limiting, at least for low levels of DNA damage (Figure 3D). The increased HO DSB ChIP signal of Mre11-W248R implies that it forms a complex with Rad50 that properly localizes in the nucleus.

**Resolution-dependent checkpoint signaling defect in mre11-W248R mutant**

DSB resection leads to formation of ssDNA coated with RPA, which recruits Rad3XRad26XRPA complex, which then phosphorylates Chk1 kinase (39,40). Accordingly, mutations that impair DNA end processing also reduce Chk1 phosphorylation in response to DNA damage (10,15,30). As our data indicated a DSB-processing defect in mre11-W248R cells, we investigated Chk1 phosphorylation. Immunoblotting revealed a substantial defect in IR-induced formation of phospho-Chk1 in mre11-W248R cells (Figure 4A and B). This defect was similar to that in mre11-H134S nuclease dead cells. IR-induced Chk1 phosphorylation was not significantly decreased in N122S and W215C mutants, which is consistent with their resistance to DNA damage (Figure 3A).

Notably, there was a small increase of Chk1 phosphorylation in mre11A, mre11-W248R and mre11-H134S cells detected before IR treatment (Figure 4A), which is consistent with the increase in spontaneous Rad52 foci and elongated cells (Figure 3B and Supplementary Figure S3) in these mutants. It is likely that defects in repair of spontaneous DNA damage results in sustained checkpoint activation in a subset of cells.

**mre11-W248R mutant fails to restore collapsed replication forks**

HR proteins, including the MRN complex and Ctp1, are essential to restore collapsed replication forks, which are a primary endogenous source of DSBs (28,41). To address whether mre11-W248R cells can repair collapsed replication forks, we tested for genetic interactions with rad2Δ, which eliminates the FEN1/Rad27 flap endonuclease ortholog that is required for Okazaki fragment processing (42). Unprocessed Okazaki fragments collapse replication forks, creating a critical requirement for HR repair. Tetrad dissections revealed a synthetic lethal interaction between rad2Δ and mre11-W248R, as was also observed with mre11A and mre11-H134S (Figure 4C). These data indicate that DNA end-processing activities of Mre11 are essential to restore collapsed replication fork by HR repair.

**Meiotic defect of mre11-W248R mutant**

Mre11 is essential for repair of DNA ends that have covalently attached proteins, such as when Rec12 (Spo11) generates programmed DSBs in meiosis. Rec12 removal from the 5' DNA end requires Mre11 endonuclease activity and Ctp1 (43-47). To assess the effect of Mre11-W248R in meiosis, we crossed opposite mating types and analysed ascus morphology and spore viability. Asci from a wild-type mating contained four evenly sized spores (Figure 4D). In contrast, matings with mre11-W248R, mre11A and mre11-H134S backgrounds produced asci with aberrant spore morphology, varying in shape and size, which is indicative of unequal chromosome segregation. Accordingly, spore viability in the mutants was drastically reduced (Figure 4D), indicating that these mutations prevent proper meiosis.

**Eliminating Ku suppresses Mre11-W248R**

Ku complex, consisting of the Ku70/Ku80 heterodimer, binds DSBs to promote non-homologous end joining repair. Eliminating Ku can partially rescue MRN and Ctp1 mutants (8,10,28,48-50). This rescue depends on Exo1 exonuclease, which can replace the MRN-Ctp1 resection activity but relies on it to displace Ku from DNA ends. We found that the slow growth and DNA damage sensitivity of the mre11-W248R mutant is also partially rescued by eliminating Ku (Figure 4E), indicating that Mre11-W248R is unable to efficiently initiate DNA end processing in vivo. This rescue requires Exo1, which also plays an important role in the extended resection of DNA ends required for homologous recombination (10). Accordingly, mre11-W248R mutants in combination with exo1Δ result in a substantial decrease in cell viability.
both prior to and after treatment with DNA damaging agents. This is consistent with our previous observation with mre11Δ and mre11-H134S cells (8), and reflects the requirement of the nuclease activity of the MRN complex in concert with Exo1 in the early steps of HR repair of DNA damage.

**mre11-W248R mutation alters interaction with Rad50**

Mre11-N117S and W210C mutations impair co-immunoprecipitation (co-IP) of Mre11 with Nbs1 in mammalian cells (19,20,51). To investigate the effects of SpMre11-W248R, we performed reciprocal co-IP experiments between MRN subunits. As expected, wild-type Mre11 and Nbs1 efficiently and reciprocally co-IP (Figure 5A). In contrast, reciprocal Mre11-Nbs1 co-IPs were strongly diminished in Mre11-N122S and -W215C backgrounds, even though these mutations did not cause sensitivity to DNA damage (Figure 3A). In the Discussion section, we address how mutations can impair co-IPs without causing DNA repair defects. The reduced interactions of Mre11-N122S and W215C with Nbs1 are consistent previous data (36) and with structural evidence indicating that these residues may impact Nbs1 interaction regions (32). These mutations did not affect co-IPs with Rad50 (Figure 5B). Co-IPs also showed that Mre11-W248R similarly reduced interactions with Nbs1 (Figure 5C). However, in contrast to Mre11-N122S and W215C, Mre11-W248R also reduced co-IP efficiency with Rad50 (Figure 5D). We also examined Mre11-CL454RR CV479RR (Mre11-RRRR), which mutates the Helix-Loop-Helix (HLH) motif near the C-terminus of Mre11 (Figure 1B) that forms the dominant Mre11-Rad50 interaction interface (34,35,52,53). As predicted by the Mre11-Rad50 structures, we were unable to co-IP
Mre11-RRRR with Rad50 (Figure 5D), which correlates with the acute sensitivity of mre11-RRRR to DNA damaging agents (52). Our results indicate that mutations that model ATLD alleles decrease stability of the Mre11-Nbs1 interactions as assayed by co-IPs, but that SpMre11-W248R is unique in that it also impairs interactions with Rad50.

Tel1 (ATM) checkpoint signaling is unaffected by Mre11-W248R

Along with its functions in DNA repair, the MRN complex also recruits the checkpoint kinase ATMTel1 to DNA lesions through interactions with the C-terminus of Nbs1 (3,54,55). ATMTel1 activity therefore depends on the assembly of MRN complex and its capacity to bind DSBs. Thus, phosphorylation of ATMTel1 substrates provides an in vivo readout for these events. The best characterized substrate of Tel1 in fission yeast is histone H2A (analogous to H2AX in mammals), which is also phosphorylated by Rad3ATR (56,57). Therefore, Tel1 activity can be specifically assayed by performing experiments in a rad3Δ background. As expected, IR-induced formation of phospho-H2A (γH2A) was partially maintained in rad3Δ and tel1Δ single mutants but was abolished in the rad3Δ tel1Δ double mutant (Figure 6A and B). γH2A was absent in mre11Δ rad3Δ cells, showing that Mre11 is required for Tel1 activity at DSBs (3). Importantly, γH2A was formed in mre11-W248R rad3Δ cells (Figure 6A and B). Thus, despite the co-IP data indicating destabilized interactions amongst the MRN subunits in lysates from mre11-W248R cells, our in vivo functional assays indicate that the MRN complex is sufficiently stable to recruit Tel1 to DSBs.

Mre11-W248R retains telomere maintenance functions

The MRN complex also recruits Tel1 to chromosome ends, where it shares an overlapping activity with Rad3 in maintaining telomeres (2,58–61). Therefore, to independently assess whether the mre11-W248R mutation...
affects the ability of the MRN complex to bind DNA ends and recruit Tel1, we performed Southern blot analysis of genomic DNA isolated from *S. pombe* and probed for telomere associated sequences (TAS1) (31). Our results demonstrate that unlike the *tel1Δ rad3Δ* or *mre11Δ rad3Δ* strains, which showed no signal, the *mre11*-W248R *rad3Δ* had a TAS1 signal, indicating the presence of intact telomeres (Figure 6C). The same result was observed for *mre11-H134S rad3Δ* cells. From these results, we conclude that Tel1 can localize to telomeres and is active in the *mre11*-W248R and *mre11-H134S* backgrounds.

Reduced Ctp1 localization at a DSB in *mre11*-W248R cells

Collectively, these data suggest that the *mre11*-W248R mutation disturbs interactions amongst the MRN subunits that are required for efficient DNA end processing, but it retains interactions that are sufficient for recruiting Tel1 to perform checkpoint signaling and telomere maintenance functions. DNA end processing by MRN complex requires Ctp1, whereas Tel1 activity is independent of Ctp1. Indeed, Tel1 checkpoint signaling is increased in *ctp1Δ* cells because of increased retention of MRN and Tel1 at DSBs (30). To address whether interactions with Ctp1 are disturbed by the *mre11*-W248R mutation, we performed ChIP analysis at the HO DSB site. This analysis revealed that the Ctp1 signal at HO DSB in *mre11*-W248R cells was reduced compared with wild-type (Figure 6D). This occurred even though Mre11 had the opposite pattern—its signal at the DSB was increased in *mre11*-W248R cells (Figure 3D). These data indicate that an inability to efficiently recruit Ctp1 contributes to the DNA end-processing defect in *mre11*-W248R cells.
DISCUSSION

In this study, we have investigated the properties of Mre11-W243R, a mutation found in two brothers with a particularly severe form of ATLD. Unlike prior cases of ATLD, these ATLD17/18 patients also developed cancer. The question arises as to how the biochemical consequences of Mre11-W243R differ from other ATLD alleles.

X-ray crystallography studies of SpMre11 bound to a portion of Nbs1 predicts that the equivalent residue Trp248 may affect both nuclease activity and Nbs1 binding, as it forms the hydrophobic core of the structural region, linking the nuclease active site to an Nbs1 contact site (32). Our data establish that HsMre11-W243R retains full in vitro DNA nuclease activities when assayed without the other MRN subunits (Figure 1). In this regard, W243R differs from mutations that directly alter critical residues in the nuclease active site, such as mutations corresponding to HsMre11-H129N or SpMre11-H134S, which have severe effects in mice and fission yeast (8,15).

Our new X-ray crystal structure and biochemical assays show that the mutation in PmMre11 analogous to W243R does not alter the architecture of the highly conserved active site but rather impairs nuclease activity (Figure 2), providing insight as to why HsMre11-W243R also retains nuclease activity. However, our in vivo studies with SpMre11-W248R indicate defects in DNA end processing, which are likely explained by an inability to form a fully functional complex with Rad50, Nbs1 and Ctp1, which is required for DNA end processing in vivo.

The structure of SpMre11-Nbs1 has also shed light on the effect of ATLD mutations on Mre11 binding to Nbs1. Mre11-N122S derived from ATLD3/4 maps to eukaryotic specific loops bridging the Mre11 dimers that make direct contact with the Nbs1-NFKxFSK motif (32). Mre11-W215C (ATLD7/8) and Mre11-W248R (ATLD 17/18) are predicted to alter structural elements that affect the critical Nbs1 interaction region along the side of the Mre11 nuclease core. These mutations all destabilized Mre11 binding to Nbs1 when assessed using purified proteins in gel filtration assays in the absence of Mn2+ (32).

Interestingly, the repair defects of budding yeast mre11-N113S cells were attributed to defects in nuclear localization that could be rescued by the fusion of a nuclear localization signal (32). This is unlikely to be the case for the analogous allele in S. pombe, as mre11-N122S cells are largely insensitive to DNA damaging agents (Figure 3A) (36). A nuclear localization defect is also unlikely to explain mre11-W248R phenotypes, as our ChIP data showed efficient binding to a DSB (Figure 3D). Indeed, the persistent binding of SpMre11-W248R at a DSB is especially informative, as it is strikingly similar to mutations that impair the DNA end-processing activity of MRN complex without destroying the overall integrity of the complex. Notably, we have observed that the Mre11-H134S nuclease-dead allele has these properties (5). Moreover, as observed with Mre11-W248R, we found that the H134S mutation causes strong sensitivity to DNA damaging agents, an acute meiotic defect and synthetic lethality with a mutation that eliminates Rad2 (FEN1), without blocking the ability of the MRN complex to recruit active Tel1ATM to DSBs, or to maintain telomeres. This constellation of effects is seen with elimination of Ctp1, which is essential for the DNA end-processing activity of the MRN complex in vivo (10,28,30).

Our histone H2A phosphorylation and telomere maintenance assays established that Tel1 ATM activity is maintained in mre11-W248R cells (Figure 6). We performed these assays in cells lacking Rad3ATR, which allowed us to specifically assess the activity of Tel1. From these results, we conclude that components of the MRN complex interact sufficiently well in mre11-W248R cells to form a complex that can bind DSBs and recruit Tel1ATM. The same results were obtained with the nuclease-dead mre11-H134S allele in the rad3A background (Figure 6C) (30), which is consistent with studies in mammalian cells indicating that Mre11 nuclease activity is dispensable for ATM activation in response to DNA damage or telomere deprotection (15).

The synthetic lethality of Mre11-W248R with a mutation that eliminates Rad2 (FEN1) supports their complementary roles in protecting or processing forks and is consistent with recent results showing Mre11 can protect or degrade stalled forks in human cells depending on BRCA1/2 partner status (41,62,63). Both Mre11 and FEN1-DNA complex structures indicate that DNA is unpaired for endonuclease cleavage, and FEN1 structures imply that correct positioning of DNA duplex is critical not only for specific DNA opening and nuclease activity, but also for productive interaction with protein partners (64).

Unlike other alleles that model ATLD mutations, the mre11-W248R allele causes acute sensitivity to DNA damage, increased spontaneous Rad52 foci, synthetic lethality with loss of Rad2/FEN-1, a strong defect in Chk1 activation in response to IR and an inability to complete meiosis (Figures 3 and 4). Yet, Mre11-W248R associates with DSBs in vivo and forms an MRN complex that recruits active Tel1ATM (Figures 3 and 6). In these respects, Mre11-W248R is very similar to the H134S nuclease dead allele; yet, Mre11-W248R clearly retains
its intrinsic nuclease activities. What then accounts for the mre11-W248R phenotypes? One clear distinction between W248R and the other ATLD alleles (i.e. N122S and W215C) is the defective co-IP with Rad50 (Figure 5). Recent structures of Mre11-Rad50 in bacterium and archaea have shown that ATP binding and hydrolysis induces large conformational changes that have implications on the nuclease activity of Mre11 (34,35,53). ATP hydrolysis leads to the ‘opening’ of the MR complex, exposing the nuclease sites of Mre11 to DNA through conformational changes that cause an interaction interface to form between the helix αF in the cap domain of Mre11 and the lobe of Rad50 (34,35). Interestingly, our structure of PfMre11-L204R causes the misfolding of αF (loop L3) required for this interface (Figure 7). Although it remains to be shown whether the Mre11-Rad50 conformational changes or the misfolding of loop L3 are conserved in the eukaryotic MRN complex, this would explain the compromised interaction we observed between Mre11-W248R and Rad50 by co-IP. An inability to properly interface with Rad50, or to correctly position DNA, could explain why Mre11-W248R mutants are proficient for binding DNA damage sites and enabling Tel1 signaling, yet shows defects in DNA end processing (Figure 7). Fittingly, mutations that perturbed the interaction between Methanococcus jannaschii Mre11 cap domain and Rad50 lobe result in decreased endonuclease activity (53).

Our data also suggest that defective interactions of the MRN complex with Ctp1 may contribute to the mre11-W248R phenotypes. Ctp1 localization at HO DSB is decreased in these cells, even though Mre11 retention is increased (Figures 3D and 6D). Furthermore, the Ctp1 signal at HO DSB is actually increased in nuclease-defective mre11-H134S cells, which otherwise have a spectrum of phenotypes that are similar to mre11-W248R cells (5). Defective interactions between Mre11 and Nbs1 could explain defects in Ctp1 localization at a DSB in mre11-W248R cells, as Ctp1 recruitment requires an interaction with Nbs1 (4,5), but Tel1 activity also requires an interaction with Nbs1 (54,55), and our data show that Tel1 signaling is intact. It is possible that
efficient recruitment of Ctp1 to DSBs requires interactions with both Mre11 and Nbs1.

The acute DNA damage-sensitive phenotypes caused by the mre11-W248R mutation in fission yeast raises the question of whether the equivalent W243R substitution has comparable effects in humans. The structural and mechanistic effects are likely similar, although the W243R mutation may have weaker consequences in the context of the human MRN complex. It is also formally possible that the g.2499 G>A allelic which eliminates 27 residues in the cap domain, is a milder hypomorphic allele, although structural considerations predict the opposite.

The increased foci of Rad52 (which has parallel functions to human BRCA2) and inability to repair collapsed replication forks in mre11-W248R cells are strongly indicative of genome instability in fission yeast. Although it remains to be established whether the exceedingly rare pediatric cancers in ATLD17/18 patients were directly connected to the Mre11 deficiencies, the correlations are nonetheless striking and support a role for Mre11 in preventing cancer-causing genome instability, as was first demonstrated for ATM and Nbs1.

ACKNOWLEDGEMENTS

We thank Sari van Rossum-Fikkert for help with production and purification of human protein and Taema Bajo for technical support. We acknowledge Yoshiki Yamada, Santiago Cavero, Nicholas Rhind and Devon Nieto for technical support. We acknowledge Sari van Rossum-Fikkert for help with production and purification of human protein and Taema Bajo for technical support. We acknowledge Sari van Rossum-Fikkert for help with production and purification of human protein and Taema Bajo for technical support. We acknowledge Sari van Rossum-Fikkert for help with production and purification of human protein and Taema Bajo for technical support.

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