A Mutant Allele of *MRE11* Found in Mismatch Repair-deficient Tumor Cells Suppresses the Cellular Response to DNA Replication Fork Stress in a Dominant Negative Manner

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The interaction of *ataxia-telangiectasia mutated* (ATM) and the Mre11/Rad50/Nbs1 (MRN) complex is critical for the response of cells to DNA double-strand breaks; however, little is known of the role of these proteins in response to DNA replication stress. Here, we report a mutant allele of *MRE11* found in a colon cancer cell line that sensitizes cells to agents causing replication fork stress. The mutant Mre11 weakly interacts with Rad50 relative to wild type and shows little affinity for Nbs1. The mutant protein lacks 3'-5' exonuclease activity as a result of loss of part of the conserved nuclease domain; however, it retains binding affinity for single-stranded DNA (ssDNA), double-stranded DNA with a 3' single-strand overhang, and fork-like structures containing ssDNA regions. In cells, the mutant protein shows a time- and dose-dependent accumulation in chromatin after thymidine treatment that corresponds with increased recruitment and hyperphosphorylation of replication protein A. ATM autophosphorylation, Mre11 foci, and thymidine-induced homologous recombination are suppressed in cells expressing the mutant allele. Together, our results suggest that the mutant Mre11 suppresses the cellular response to replication stress by binding to ssDNA regions at disrupted forks and impeding replication restart in a dominant negative manner.

INTRODUCTION

The MRN complex, consisting of Mre11, Rad50 and NBS1, has diverse functions in DNA damage recognition (Petrini and Stracker, 2003), DNA replication (Costanzo *et al.*, 2001), cell cycle checkpoint activation (Grenon *et al.*, 2001), nonhomologous end joining (Paull and Gellert, 2000), and telomere maintenance (Wu *et al.*, 2007). The Mre11 complex binds DNA double-strand breaks (DSBs) soon after they are formed implicating it in DNA damage detection (Mirzoeva and Petrini, 2001). Furthermore, the complex can tether linear duplex molecules (de Jager *et al.*, 2001), and it is able to bridge broken DNA ends or sister chromatids (van den Bosch *et al.*, 2003). Mre11 has 3'-5' exonuclease activity and

endonuclease activity (Paull and Gellert, 1999), suggesting a role in the processing of DNA ends into forms that can be recognized by cell cycle checkpoint and DNA repair proteins (Paull and Gellert, 1999; Lee and Paull, 2005; Jazayeri *et al.*, 2006). However, precise cellular roles of the Mre11 complex have been difficult to establish, because null mutations of all components of the complex are lethal to vertebrate cells (Luo *et al.*, 1999; Yamaguchi-Iwai *et al.*, 1999; Zhu *et al.*, 2001).

There are several lines of evidence implicating the MRN complex in DNA replication. The complex associates with chromatin and colocalizes with proliferating cell nuclear antigen (PCNA) throughout S phase (Maser *et al.*, 2001). In addition, chromatin loading of Mre11 is enhanced by fork stalling, suggesting that the complex is loaded at the replication fork (Mirzoeva and Petrini, 2003). Depletion of Mre11 from DT40 or *Xenopus* leads to increased chromosomal breaks and accumulation of DSBs during DNA replication (Yamaguchi-Iwai *et al.*, 1999; Costanzo *et al.*, 2001). There is evidence that the MRN complex is required for the restart of collapsed replication forks, and it is this function that prevents the accumulation of DNA breaks during DNA replication (Trenz *et al.*, 2006). It has been proposed that Mre11 promotes recapture of broken DNA at collapsed forks favoring the reassembly of functional forks, possibly through homologous recombination (HR)-mediated events (Trenz *et al.*, 2006). Indeed, the metabolism of secondary structures arising at replication fork is partially dependent on components of the analogous MRN complex in both *Escherichia coli* (SbcCD) and *Saccharomyces cerevisiae* (Mre11, Rad50, and

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Abbreviations used: ATM, ataxia-telangiectasia mutated; ATR, ATM and Rad3 related; CPT, camptothecin; dNTP, deoxyribonucleoside triphosphate; DSB, DNA double-strand break; dsDNA, double-stranded DNA; HR, homologous recombination; IR, ionizing radiation; MMR, mismatch repair; MRN, Mre11/Nbs1/Rad50; MSI, microsatellite instability; ssDNA, single-stranded DNA.

Xrs2) (Petrini, 2000; Lobachev *et al.*, 2002). Therefore, the essential function of the MRN complex may be in the restart of collapsed or stalled replication forks.

The phosphoinositide 3-kinase-related protein kinase (PIKK) family member, ataxia-telangiectasia mutated (ATM) is a key signal transducer of the DNA damage response. Although ATM responds primarily to agents that produce DSBs in DNA, other stimuli are also capable of ATM activation (Bakkenist and Kastan, 2003; Takai *et al.*, 2003; Bolderson *et al.*, 2004). The Mre11 complex plays an important role both in the recruitment of ATM to the sites of DNA damage and in the efficient activation of ATM (Carson *et al.*, 2003; Uziel *et al.*, 2003; Falck *et al.*, 2005; You *et al.*, 2005). MRN assembles DNA fragments into signaling centers that are required for ATM activation (Costanzo *et al.*, 2004). In addition to its roles upstream in the signaling cascade, the Mre11 complex is a substrate of ATM, suggesting other downstream functions (Gatei *et al.*, 2000).

ATM is required for the cellular response to DNA replication fork stress induced by agents such as thymidine (Bolderson *et al.*, 2004) or camptothecin (CPT, (Smith *et al.*, 1989; Pommier, 2006). Thymidine disrupts DNA synthesis and slows S phase progression by altering the balance of deoxyribonucleoside triphosphates (dNTPs) (Bjursell and Reichard, 1973). In the presence of thymidine, the pool of dTTP expands and reduces the supply of dCTP through the allosteric inhibition of ribonucleotide reductase. CPT specifically traps the topoisomerase I cleavage complex that normally transiently forms to relax supercoiling generated during DNA replication, and the collision of replication forks with this topoisomerase I cleavage complex produces a collapsed replication fork (Pommier, 2006). We have reported that a subset of human colon cancer cell lines defective in mismatch repair (MMR) are thymidine sensitive and defective in some forms of HR (Mohindra *et al.*, 2002), whereas others (Jacob *et al.*, 2001) have shown sensitivity to CPT in some of these lines. To determine whether the sensitivity of tumor cell lines to such replication inhibitors is the result of a defect in the ATM-mediated response to replication fork stress we determined the integrity of this damage response pathway. Here, we report a mutant allele of *MRE11* found in the MMR-deficient tumor cell line HCT116. This mutant allele confers sensitivity to both thymidine and CPT, shows impaired binding to NBS1 and Rad50 and suppresses ATM activation in response to replication stress. The mutant Mre11 retains the ability to bind DNA but has defective 3'-5' exonuclease activity, suggesting that processing of replication intermediates in cells expressing this mutant might be impaired.

MATERIALS AND METHODS

Cell Lines and Culture

Human embryonic kidney 293 cells, SW480, and HCT116 were obtained from American Type Culture Collection (Manassas, VA). Derivatives of SW480 and HCT116 containing the *Scneo* recombination reporter (SW480/SN3 and HCT116/HN5, respectively) were described previously (Mohindra *et al.*, 2002). All cells were maintained in DMEM supplemented with 10% fetal calf serum. Cytotoxic responses to thymidine, CPT, and ionizing radiation (IR) were performed as described previously (Mohindra *et al.*, 2002). All experiments were performed independently three to five times.

Expression Constructs and Transfections

Wild-type Mre11 and Δ_{5-7} Mre11 were amplified from MRC5 and HCT116 cells, respectively by reverse transcription-polymerase chain reaction and cloned into pIRESpuo3 (Clontech, Mountain View, CA). FLAG-tagged MRE11s were amplified from these constructs by using a primer containing a C-terminal FLAG sequence. Rad50 and Nbs1 cDNAs were amplified from MRC5 cells and cloned into pIRESpuo3. cDNAs were sequenced to ensure

their authenticity. Expression constructs were transfected into SW480/SN3 or 293 by using Lipofectamine (Invitrogen, Paisley, United Kingdom). Colonies obtained were expanded in puromycin-selective medium (0.7 μ g/ml), and they were characterized by sequencing and Western blotting.

Immunoblotting and Immunoprecipitation

Immunoblotting was performed as described previously (Bolderson *et al.*, 2004). Antibodies used were rabbit polyclonal phospho-Ser1981-ATM (Abcam, Cambridge, United Kingdom), ATM (Calbiochem, San Diego, CA), phospho-Ser345-CHK1 (Cell Signaling Technology, Danvers, MA), MRE11 (Cell Signaling Technology), NBS1 (Oncogene), phospho-Ser343-NBS1 (Cell Signaling Technology), Rad50 (Bethyl Laboratories, Montgomery, TX), ORC2 (BD PharMingen), β -actin (Sigma Chemical, Polle, Dorset, United Kingdom), and mouse anti-FLAG (Sigma Chemical). Anti-rabbit (Cell Signaling Technology) or anti-mouse (Cell Signaling Technology) horseradish peroxidase conjugates were used as a secondary antibody. Immunoreactive protein was detected using SuperSignal West Pico chemiluminescence substrates (Pierce Chemical, Rockford, IL), and it was visualized by Las 3000 (Fujifilm, Tokyo, Japan) or x-ray film (Fuji) exposure.

Cells expressing Mre11-FLAG or Δ_{5-7} Mre11-FLAG were transfected with Rad50 or Nbs1 constructs, and they were harvested after 16 to 24 h with lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 0.5% Triton X-100). Supernatants obtained following centrifugation at $12,000 \times g$ for 10 min were treated with FLAG M2 affinity gel (Sigma Chemical) at 4°C for 3–4 h. Pellets were washed three times with Tris-buffered saline (TBS) buffer (50 mM Tris-HCl and 150 mM NaCl, pH 7.4) to remove unbound proteins. For Nbs1 or Rad50 immunoprecipitations, cell lysates were incubated with antibodies in the presence of protein A agarose beads (Calbiochem) for 2 h at 4°C. Precipitates were boiled 3 min in SDS loading buffer, and they were analyzed by Western blotting.

Mre11/ Δ_{5-7} Mre11 Expression and Purification

Expression constructs for C terminal FLAG-tagged wild-type and mutant Mre11 were transfected into 293 cells by using Lipofectamine (Invitrogen), and they were allowed to grow for 48–72 h. Cell lysates were prepared and incubated with FLAG M2 affinity gel suspension (Sigma Chemical) at 4°C overnight as recommended by the manufacturer (Sigma Chemical). The affinity gel was collected and washed with TBS (10 column volumes), followed by TBS containing 0.5 M LiCl (4 column volumes), and TBS (10 column volumes). Bound proteins were eluted using FLAG peptides (100 μ g/ml) (Sigma Chemical), and they were analyzed by Western blotting. Fractions containing Mre11 were dialyzed against buffer A (20 mM Tris-HCl, pH 8, 1 mM EDTA, 0.5 mM dithiothreitol, and 10% glycerol), and a long-term storage buffer (buffer A in 50% glycerol). Aliquots of purified proteins were kept at -80°C . The purity of the preparations was assessed using Coomassie Blue and silver-stained gels. Other components of the MRN complex were identified in preparations of the wild-type Mre11 by matrix-assisted laser desorption ionization/time of flight and Western blotting, although these were present at much lower levels. A low level of Rad50 but not Nbs1 was found in preparations of the mutant Mre11.

DNA Binding and Exonuclease Assay

5' [^{32}P]-ATP-labeled linear substrates used in DNA binding assays were 70-base pair duplex DNA, duplex DNA with a 45-base pair single-stranded DNA (ssDNA) overhang, or 45-base pair ssDNA. Oligonucleotide sequences are provided in Supplemental Material, and substrates were prepared as described previously (Castella *et al.*, 2006). Fork-like substrates consisted of a 20-base pair duplex with 45-base pair arms that were completely duplexed or contained 10- or 30-bp ssDNA gaps (see Supplemental Figure 1 for sequences of these substrates).

Binding of Mre11/ Δ_{5-7} Mre11 to forks and linear double-stranded DNA (dsDNA) substrates was measured using a gel-shift assay (Mahdi *et al.*, 2003). Various concentrations of protein and 1 nM ^{32}P -labeled DNA substrate were mixed in gel binding buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1 mM dithiothreitol, 100 g/ml bovine serum albumin, and 6% [vol/vol] glycerol), and the mixture was incubated on ice for 15 min before loading onto a prechilled 4% native polyacrylamide gel in low ionic strength buffer (6.7 mM Tris-HCl, pH 8.0, 3.3 mM sodium acetate, and 2 mM EDTA). Electrophoresis was carried out at 160 V for 90 min at room temperature. Gels were dried, and then they were analyzed with a Fujifilm FLA3000 PhosphorImaging system. Exonuclease assays were performed as described previously (Paull and Gellert, 1999).

Chromatin Fractionation

Cells were fractionated into cytosolic (S1), soluble nuclear (S2), and chromatin-bound fractions as described previously (Li and Stern, 2005).

Mre11 Foci

Poly-L-lysine-coated coverslips from treated and untreated cultures were processed as described by Maser *et al.* (2001). These coverslips were then incubated with rabbit anti-Mre11 and mouse anti-FLAG antibodies followed

by fluorescein isothiocyanate-conjugated goat anti-rabbit. Cells were also 4,6-diamidino-2-phenylindole stained. Images were captured using a Quantix camera (Photometrics, Tucson, AZ), and gray scale images were processed using Openlab and Velocity software (Improvision, Coventry, United Kingdom).

Recombination Assays

Recombination assays were performed as described previously (Bolderson *et al.*, 2004). For recombination induced by thymidine, replica cultures of SW480/SN3 cells were treated for 24 h with various doses of thymidine, and then they were allowed to recover in complete medium for 2 d before plating in 1 mg/ml Geneticin (G418; Invitrogen) (at a density of 13–25 cells/mm²). After 12–14 d, plates were stained, and colonies with >50 cells were scored. Recombination frequencies were calculated by dividing the number of colonies on the test plates by the total number of cells plated. This frequency was corrected for differences in viability between treated and untreated cultures by dividing the frequency of recombinants by the plating efficiency obtained in the absence of neo selection. All experiments were repeated independently two to four times. Dose-response data were analyzed by multiple linear regression analysis by using STATA statistical software (Stata, College Station, TX). Loge recombination frequency was the dependent variable, and thymidine dose and cell line were the independent variables. The contribution of the cell line variable to recombination frequency was determined by likelihood ratio test for the comparison of the linear regression model with and without that variable. Plots of residuals and fitted values were used to check the assumptions of linearity and constant variance of the error term.

RESULTS

A Mutant Allele of MRE11 Confers Sensitivity to Thymidine and CPT

To determine whether the thymidine and CPT sensitivity of MMR-deficient tumor cells is caused by mutations of genes encoding proteins involved in the ATM-mediated cellular response, we amplified and sequenced cDNAs encoding Chk2, Nbs1, Rad50, and Mre11. Although sequences for Nbs1, Rad50, or Chk2 in our tumor cell lines were identical to those of the wild-type cDNAs deposited in the databases, analysis of the Mre11 cDNA obtained from HCT116 revealed novel transcripts. Sequence analysis of one of these revealed that it lacked exons 5–7 of the 20 exons encoding Mre11 (Figure 1A) as an apparent result of a previously reported –1 or –2 frameshift in a run of 11 thymine residues in intron 4 of the *MRE11* gene found in HCT116 and a high proportion of colon cancers showing microsatellite instability (Giannini *et al.*, 2002). The mutant transcript (called Δ_{5-7} Mre11) contains an open reading frame that encodes a 593-amino acid (aa) protein compared with the 708 aa of the wild-type gene (Figure 1B). Interestingly, the Δ_{5-7} Mre11 mutation eliminates the third and fourth conserved phosphoesterase motifs that are necessary for nuclease activity (Sharples and Leach, 1995; Bressan *et al.*, 1998).

To determine whether Δ_{5-7} Mre11 contributes to thymidine and CPT sensitivity, expression constructs were transfected into the thymidine resistant colon cancer cell line SW480/SN3 and a C-terminal FLAG-tagged construct of Δ_{5-7} Mre11 was transfected into 293 cells. Two independent transfectant clones were isolated from each parental cell line (SM1.3 and SM1.6 from SW480/SN3 and FD2.11 and FD40 from 293 cells). Western blot analysis of lysates prepared from these transfectants revealed a novel protein migrating more rapidly than Mre11 that was recognized by two different Mre11 antibodies (Figure 1C; data not shown). Furthermore, the more rapidly migrating protein in extracts of FD40 and FD2.11 was detected by anti-FLAG antibody. A faster migrating form of Mre11 was detected in extracts prepared from HCT116, although the levels of both wild type and mutant Mre11 were low in extracts of this line. Because this protein was approximately the same molecular weight as the protein predicted to be encoded by Δ_{5-7} Mre11 (71 kDa), it interacted with two different Mre11 antibodies, and it has

not been detected in Western blots prepared from any of our MMR-proficient cell lines, we concluded that it represented the mutant Mre11 protein. In SM1.3 and 1.6, the mutant protein was found at a lower level than the wild type, whereas FD40 and FD2.11 showed nearly equal levels of mutant and wild-type proteins. The cause of the different expression patterns in the transfectants is unknown. Notably, all transfectants expressing Δ_{5-7} Mre11 grew more slowly, and they had a lower plating efficiency than parental cells.

To determine whether Δ_{5-7} Mre11 conferred sensitivity to thymidine and CPT, the survival of the parental lines (SW480/SN3 and 293), transfectants, and HCT116 in these agents was determined. Like HCT116 and AT cells, transfectants expressing Δ_{5-7} Mre11 were distinctly more sensitive to thymidine and CPT (Figure 1D). Given the increased sensitivity of cells from ataxia-telangiectasia-like disorder (ATLD) patients that carry hypomorphic mutations of *MRE11* to IR (Stewart *et al.*, 1999), we also examined sensitivity to this agent. Cells expressing Δ_{5-7} Mre11 showed no significant alteration in their sensitivity to IR relative to the parental cells at lower exposures. SM1.3 and 1.6 were slightly but significantly ($p = 0.0042$ for SW480/SN3 vs. SM1.3 or SM1.6 at 8 Gy by the *t* test) more sensitive at higher doses (Figure 1D). FD40 and FD2.11 were also sensitive to higher doses of IR. At 6 Gy, the survival of FD40 was $<2.7 \times 10^{-4}$, and for FD2.11 it was $<9.6 \times 10^{-5}$ after correcting for colony formation by untreated control cells, whereas the survival of parental 293 cells was 0.2%. Thus, the mutant Mre11 confers sensitivity to thymidine, CPT, and high doses of IR.

Interactions of Δ_{5-7} Mre11 with Rad50 and Nbs1 Are Impaired

Previous studies suggest that the N terminus of Mre11 is essential for Nbs1 interactions (Desai-Mehta *et al.*, 2001). To determine whether the loss of the conserved domain of Mre11 affected the formation of the MRN complex, we examined the integrity of this complex in cells expressing the mutant gene. 293 cells expressing C-terminal FLAG-tagged expression constructs for the wild-type (FW1) or mutant Mre11 (FD2.11) were transfected with expression constructs for Rad50 or Nbs1, and after 24 h the transfected cultures were harvested. FLAG immunoprecipitates obtained from lysates of these cells were then analyzed by Western blotting. Rad50 coimmunoprecipitated with full-length Mre11 and Δ_{5-7} Mre11; however, the interaction with Δ_{5-7} Mre11 was weaker (Figure 2A). Nbs1 coimmunoprecipitated with Mre11-FLAG, but it was barely detectable in immunoprecipitates with Δ_{5-7} Mre11-FLAG (Figure 2B). In addition the mutant Mre11 did not interact with the wild-type protein. Mre11-FLAG and Δ_{5-7} Mre11-FLAG were transiently expressed in 293, and extracts prepared from these cells were immunoprecipitated with anti-Rad50 or anti-Nbs1 antibodies. Western blots of these immunoprecipitates were then probed with anti-Mre11 antibody (Figure 2C). These blots revealed that both Rad50 and Nbs1 predominantly interacted with the wild-type Mre11. Thymidine treatment of the transfected cells had no effect on these interactions. Thus, the interactions of Δ_{5-7} Mre11 with both Rad50 and Nbs1 are altered.

Δ_{5-7} Mre11 Is Exonuclease Deficient but Retains DNA Binding Activity

The nuclease activity of Mre11 is essential for its ability to process DNA damage and trigger signaling in response to DSBs (Falck *et al.*, 2005). Because Δ_{5-7} Mre11 disrupts the

Figure 1. A mutant allele of *MRE11* found in HCT116 (Δ_{5-7} Mre11) eliminates a conserved domain and confers sensitivity to thymidine and CPT. (A) A splice variant of the *MRE11* cDNA found in HCT116 eliminates exons 5–7 (in *italics*) and produces an in-frame message. (B) The Mre11 protein has five motifs (I–V) in a conserved nuclease domain. Δ_{5-7} Mre11 encodes a 593-amino acid protein that lacks the third and fourth phosphoesterase motifs. (C) Immunoblots prepared from whole cell extracts of SW480/SN3, HCT116, 293, and derivatives containing an expression construct for Δ_{5-7} Mre11 (SM1.3, SM1.6, FD40, and FD2.11) probed with an antibody against Mre11. FD40 and FD2.11 contain a C-terminal FLAG-tagged Δ_{5-7} Mre11 that is detected by anti-FLAG antibody. The β -actin loading controls are presented. (D) Sensitivity of cells expressing Δ_{5-7} Mre11 to thymidine (TdR), IR, and CPT. Top, response of parental SW480, SM1.3, SM1.6, and HCT116. Bottom, sensitivity of parental 293 and strains expressing Δ_{5-7} Mre11 (FD40, FD2.11). The cells in all these dose response experiments were treated in duplicate and the experiments were performed two to four times independently. Error bars indicate standard deviations.

Figure 2. Δ_{5-7} Mre11 shows altered interactions with Nbs1 and Rad50. (A and B) Coimmunoprecipitations were carried out using FLAG M2 affinity gel. Lysates were prepared from indicated cell lines expressing C-terminal FLAG-tagged wild-type (FW1) or mutant (FD2.11) Mre11 transiently transfected with Rad50 (A) or Nbs1 (B) expression constructs. Western blots of immunoprecipitates were probed with the indicated antibodies. (C) 293 cells were transfected with C-terminal FLAG-tagged Mre11 or Δ_{5-7} Mre11 expression constructs. Whole cell lysates were incubated with anti-Rad50 or anti-Nbs1 antibodies, and immunoprecipitated proteins were analyzed by Western blotting using anti-Mre11 antibody. Western blots were also probed with the anti-FLAG antibody to measure the level of FLAG-tagged Mre11 proteins. In one culture 293 cells transfected with the expression construct for the mutant Mre11 were treated with 10 mM thymidine for 24 h.

conserved nuclease domain of Mre11, we next assayed affinity-purified preparations of mutant and wild-type Mre11 for 3'-5' exonuclease activity. Using the indicated duplex DNA as substrate, exonuclease activity was evident in assays of wild-type Mre11 (Figure 3A, lanes a-e) but not the mutant protein (Figure 3A, lanes f-j).

We next determined whether the defective exonuclease activity of Δ_{5-7} Mre11 was the result of a reduced affinity of the protein for various DNA substrates. Affinity-purified

wild-type and mutant Mre11 were incubated with three linear DNA substrates to assay binding activity in gel mobility shift assays. At the highest concentrations of the wild-type protein, bands with slower mobility relative to the free DNA were detected (Figure 3B). The mutant Mre11 forms complexes with similar mobility to those formed by the wild type with all three DNA substrates. In the absence of Mg^{2+} , it binds to dsDNA about as well as wild-type Mre11 (compare lanes f-j with f'-j'), whereas it seems to have higher binding affinity for ssDNA (lanes a-e and a'-e') and dsDNA with 3' ssDNA tail (lanes k-o and k'-o'). Addition of Mg^{2+} did not enhance the poor binding affinity of the wild-type or mutant Mre11 for dsDNA (Figure 3C). Mg^{2+} significantly improved the binding of the wild-type Mre11 to ssDNA, and in the presence of Mg^{2+} , both proteins showed similar affinity to ssDNA. Wild-type and mutant proteins show increased binding to the dsDNA with a 3' ssDNA tail, with the mutant Mre11 binding significantly better than the wild-type protein in the presence or absence of Mg^{2+} (Figure 3C). Addition of Mn^{2+} , EDTA, ATP or AMP-PNP had no effect on the binding activity of wild-type or mutant proteins (data not shown).

Although the above-mentioned experiments indicate that the mutant Mre11 can induce mobility shifts when incubated with substrates that potentially form at collapsed replication forks (the double-stranded DNA with a 3' single strand tail; Figure 3, B and C), we next investigated the ability of the mutant protein to bind replication fork-like structures that may form in response to disruption of DNA replication. Substrate 1 has 45-base pair duplexed arms with no ssDNA at the junction, whereas substrates 2 and 3 contained 10- and 30-base pair stretches of ssDNA, respectively (Figure 3D). In contrast to the wild-type protein (lane e), Δ_{5-7} Mre11 produced only a weak mobility shift after incubation with the substrate lacking ssDNA (lanes a-d). A slightly greater mobility shift was obtained in reactions with substrate 2 (containing 10-bp ssDNA regions; lanes f-i), although this was still not as pronounced as the shift obtained with the wild type (lane j). However, a more robust shift was obtained for the mutant Mre11 in reactions with substrate 3 containing 30-bp stretches of ssDNA (lanes k-n; also see Supplemental Figure 2). Wild-type Mre11 showed similar mobility shifts with this substrate (Figure 3D, lanes o-r, and Figure 3E). Thus, the mutant Mre11 was more responsive to increasing levels of ssDNA in these replication fork-like structures and shows greater affinity for substrates containing ssDNA regions of ≥ 30 base pairs.

Enhanced Binding of Δ_{5-7} Mre11 to Chromatin after Replication Fork Stress

The increased binding of the mutant Mre11 to DNA substrates containing single-stranded regions suggested that the mutant protein may be retained at such sites after fork stress. To test this hypothesis, we next examined the recruitment of wild-type and mutant Mre11 to chromatin in cells exposed to thymidine or CPT. Chromatin was fractionated from parental 293 or FD40 cells treated with thymidine, CPT, or IR. Proteins obtained from cytoplasmic (S1), soluble nuclear (S2), and chromatin (P) fractions were then analyzed by Western blotting. In untreated wild-type cells, most Mre11 was found in the chromatin fraction, and the level of this chromatin-bound Mre11 was not greatly affected after a 24-h treatment with up to 10 mM thymidine (Figure 4A). In FD40 cells, the localization of the wild-type Mre11 was similar to that found in 293, although there was a slight drop in cells treated with 10 mM thymidine. In contrast, most of the mutant Mre11 was found in the S1 fraction in FD40.

Figure 3. Δ_{5-7} Mre11 is deficient in 3'-5' exonuclease but not DNA binding activity in vitro. (A) Exonuclease activity of affinity purified Mre11/ Δ_{5-7} Mre11. Increasing concentrations of affinity purified wild-type (lanes a–e) or mutant (lanes f–j) proteins (0, 25, 50, 100, and 200 nM) were incubated with a ³²P-labeled dsDNA with 3' ssDNA overhang for 60 min before electrophoresis in a 10% denaturing polyacrylamide gel. In all the panels, the 5' ³²P-labeled strand is indicated by the red circle. (B) Gel mobility shift assays were performed with affinity-purified Mre11 and Δ_{5-7} Mre11 with the indicated substrates in the presence of increasing protein concentrations (0, 50, 100, 200, and 400 nM) and in the absence of Mg²⁺. (C) Binding of Mre11 or Δ_{5-7} Mre11 to three linear DNA substrates (ssDNA, dsDNA, and dsDNA with 3' ssDNA overhang) in the presence or absence of 5 mM Mg²⁺. Gel mobility shift assays were quantified, and mean values and standard deviations obtained from at least three independent experiments are presented. (D) Gel mobility shift assays performed with increasing amounts of Δ_{5-7} Mre11 (0, 250, 500, and 1000 nM, lanes a–d, f–i, and k–n) and Mre11 (1000 nM, lanes e and j or 0–1000 nM, lanes o–r). ³²P-labeled substrates were fork-like structures having 45-base pair double-stranded DNA on both arms (substrate 1) or various levels of ssDNA forming 10- (substrate 2) or 30-base pair (substrate 3) gaps on each arm. The concentration of each substrate in the assays was 1 nM, and the assay was carried out in the presence of 5 mM Mg²⁺. The 3' ends of the putative leading or lagging strands of the partial fork structures are indicated by arrows. (E) Fractions of substrates bound by Mre11 or Δ_{5-7} Mre11 in assays presented in D were quantified and mean values, and standard deviations obtained from two independent experiments are presented.

Figure 4. Chromatin-associated Δ_{5-7} Mre11 accumulates after thymidine or CPT treatment. Extracts of 293 or FD40 cells were fractionated into chromatin-bound fractions (P), soluble nuclear fractions (S2), and cytosolic fractions (S1), and they were analyzed by Western blotting. (A) Parental 293 and FD40 cells were treated for 24 h with increasing concentrations of thymidine as indicated. Western blots of proteins fractionated in S1, S2 or P fractions were probed with the indicated antibodies. ORC2 was used as a marker for chromatin-bound proteins in all of these experiments. The two arrows represent the positions of Mre11 (top) and Δ_{5-7} Mre11 (bottom). Chromatin-associated Mre11 and Δ_{5-7} Mre11 detected on Western blots obtained from thymidine-treated cultures were quantified using Image Gauge version 3.3 (Fujifilm). These measurements showed that the wild-type Mre11 increased up to twofold with increasing concentrations of thymidine, whereas the mutant Mre11 increased approximately fourfold. (B) The histogram presents the percentage of the total Mre11 bound to chromatin (measured as described above) represented by the mutant or wild-type proteins after treatment with increasing concentrations of thymidine. Results presented are the mean of at least two independent experiments. Standard deviations are presented. (C). Parental 293 and FD40 cells were exposed to 10 mM thymidine for the indicated times. Proteins isolated in the nuclear, chromatin bound, and cytoplasmic fractions were analyzed by Western blotting by using the indicated antibodies. (D) Western blot analysis of MRE11 and Δ_{5-7} Mre11 from fractionated cells treated with 250 nM CPT for the indicated times. (E) Western blot analysis of Mre11 and Δ_{5-7} Mre11 from fractionated cells treated with 2 Gy of IR. Cells were harvested at the indicated times after irradiation for analysis.

When these cells were treated for 24 h with increasing concentrations of thymidine, the level of chromatin-bound wild-type Mre11 increased up to twofold (in 5 mM thymidine), whereas the mutant Mre11 increased approximately fourfold. In untreated FD40 cells, more wild-type Mre11 was found in the chromatin fraction than mutant Mre11; however, as the concentration of thymidine increased the mutant protein became the predominant form of Mre11 bound to chromatin (Figure 4, A and B). When parental 293 cells were treated with 10 mM thymidine for varying lengths of time, there was relatively little change in the level of wild-type Mre11 in the chromatin fraction over the entire 24-h treatment (Figure 4C). In FD40 cells, there was a time-dependent accumulation of mutant, and to a lesser extent wild-type proteins. Thus, in cells expressing the mutant Mre11, the association of both mutant and wild-type proteins with chromatin is dependent upon the time and concentration of thymidine exposure. However, there is a more pronounced accumulation of Δ_{5-7} Mre11 in chromatin than the wild type protein.

The level of Nbs1 associating with the chromatin fraction in parental 293 cells showed a slight decline after treatment with higher concentrations of thymidine or longer times of exposure (Figure 4, A and C). For FD40 cells, the association of this protein with chromatin showed both time- and concentration-dependent increases that seemed to parallel the changes of the wild-type Mre11. Similarly RPA32 accumulated in chromatin of FD40 cells treated with thymidine. This accumulation along with RPA32 hyperphosphorylation was particularly evident in FD40 cells treated for 24 h (Figure 4C), suggesting that ssDNA regions forming after the disruption of DNA replication by thymidine accumulate in FD40 cells.

FD40 cells treated with CPT also show an accumulation of the chromatin bound mutant Mre11 at 24 h after treatment, whereas the level of wild-type protein decreases (Figure 4D). In contrast, FD40 cells exposed to 2 Gy of IR showed an increased level of the wild-type Mre11 over the 24 h after irradiation, whereas the mutant protein showed a weaker increase (Figure 4E), consistent with the weak effect of the mutant Mre11 on the cellular response to this agent.

Formation of Mre11 Foci Is Suppressed in Cells Expressing Δ_{5-7} Mre11

Mre11 foci form in response to IR or agents that induce replication fork stress. To determine whether the mutant Mre11 altered the formation of these foci, cells treated for 24 h with 0.5 or 10 mM thymidine were processed as described by Maser *et al.* (2001), and they were stained for Mre11. A high proportion of the parental 293 cells treated with 0.5 or 10 mM thymidine showed an increase in the level of Mre11 foci (>10 /cell; Figure 5A). The fraction of cells staining for these foci was significantly lower in FD40 cells treated with either concentration of thymidine relative to the parental line (Figure 5, A and B). Mre11 foci were also induced in a high proportion of wild-type cells by treatment with 20 nM CPT consistent with a previous report (Furuta *et al.*, 2003), whereas in FD40 cells there was no significant induction. In contrast there was a significant induction of MRE11 foci at 2 h after treatment of FD40 cells with IR (Figure 5A).

Cells Expressing Δ_{5-7} Mre11 Are Defective in the Autophosphorylation of ATM but Not Phosphorylation of Chk1, Chk2, or Nbs1 after Thymidine Treatment

We next determined whether ATM autophosphorylation was affected in cells expressing the mutant Mre11 after

thymidine treatment. Autophosphorylation of ATM at Ser1981 was detected in SW480/SN3 and derivatives expressing the mutant Mre11 (SM1.3 and 1.6) within 15 min of exposure to IR (Figure 6A). Similarly, pSer1981-ATM was detected in SW480/SN3 cells after a 15-min treatment with 0.5 mM thymidine. In contrast autophosphorylation of ATM was not detected in SM1.3 and SM1.6 cells after this treatment (Figure 6B). FD40 cells showed a similar defect in ATM autophosphorylation (Figure 6C). pSer1981-ATM was evident in parental 293 cells within 60 min of treatment with 0.5 mM thymidine, but it was not detected in FD40 cells expressing the mutant Mre11, even after a 24-h exposure. If FD40 cells were treated with a much higher dose of thymidine (10 mM), there was still no increase in the level of autophosphorylated ATM over background after a 60-min exposure (Figure 6C). The level of total ATM was not greatly altered by these treatments in any of the cells tested. Thus, Δ_{5-7} MRE11 suppressed the activation of ATM after exposure to thymidine.

We next determined whether the expression of the mutant Mre11 affected the ATM-mediated protein kinase cascade triggered by thymidine. Phosphorylated forms of Chk2 (pThr68) and Nbs1 (pSer343) were evident 15–30 min after treatment of 293 or FD40 cells with 10 mM thymidine, although their induction seemed less robust in FD40 (Figure 6C). In addition phosphorylation of Chk1 was detected 15–30 min after treatment. Thymidine concentrations as low as 0.5 mM were sufficient to trigger phosphorylation of Nbs1 after 1 h of treatment of parental 293 cells (Figure 6D). pSer343-Nbs1 was also evident in FD40 cells after similar treatments; however, again, the induction was less robust than in the parental 293. The phosphorylation of Chk1 was detected in both parental 293 and FD40 cells at 1 h after treatment with thymidine concentrations as low as 0.5 mM thymidine (Figure 6D). Thus, ATM autophosphorylation was suppressed in cells expressing the mutant Mre11, but there was little or no effect on the downstream phosphorylation of Chk2 or Nbs1 or the ATM and Rad3 related (ATR)-mediated phosphorylation of Chk1.

Cells Expressing Δ_{5-7} Mre11 Are Defective in Thymidine-induced Recombination but Not Recombination Induced by a Site-specific DSB

Previous work has shown that cells defective in HR become sensitive to thymidine and that thymidine is a potent inducer of HR (Lundin *et al.*, 2002; Mohindra *et al.*, 2002). These observations indicate that thymidine induces lesions at DNA replication forks that must be resolved by HR for cell survival. Given the increased thymidine sensitivity of cells expressing Δ_{5-7} Mre11, we next determined whether this mutant gene affected the induction of recombination by thymidine. Recombination was assayed using the SCneo reporter that measures homology-based recombination events between two defective neo resistance genes (Johnson *et al.*, 1999). Replica cultures of SW480/SN3, SM1.3, and SM1.6 cells (all containing a single copy of the recombination reporter (Mohindra *et al.*, 2002) were treated with increasing concentrations of thymidine, and then they were plated in G418 to determine the frequency of recombinants induced by the treatment. To more accurately determine the effect of thymidine on the induction of recombination, replica cultures were inoculated with 1000 cells to dilute out preexisting neo⁺ cells, and they were grown to $\sim 1 \times 10^6$ cells before a 24-h treatment with thymidine. Treated cultures were then left for 2 d to allow recovery before plating in selective medium containing G418. Using this approach, thymidine treatment increased the frequency of neo⁺ recombinants up

Figure 5. Formation of Mre11 foci after thymidine treatment is suppressed in cells expressing Δ_{5-7} Mre11. (A) Parental 293 and FD40 cells were treated with 2Gy IR before being fixed at 2 h after treatment for analysis of Mre11 foci by immunofluorescence by using anti-Mre11 antibody. Mre11 foci were also examined in these cells after 24-h treatments with 0.5 or 10 mM thymidine or a 2-h treatment with 20 nM CPT. (B) Mre11 foci in the control or treated cells were scored, and the percentages of cells containing >10 Mre11 foci were determined. Mean values and standard deviations from at least two independent experiments are presented.

to sevenfold in a dose-dependent manner in cultures of SW480/SN3 (Figure 7A). To determine whether cells expressing Δ_{5-7} Mre11 were defective in thymidine-induced recombination, cultures of SM1.3 and SM1.6 were treated with this agent. After treatment with up to 10 mM thymidine there was no increase in the frequency of neo⁺ colonies over background (Figure 7A). Comparison of the dose responses using regression analysis showed that the recombination frequency of SW480/SN3 cells had a significantly increased response to thymidine compared with the SM1.3 and SM1.6 cells (likelihood ratio test, $p = 0.0025$). Similarly treatment of HCT116 cells containing a single copy of SCneo with up to 2 mM thymidine did not result in any increase in the frequency of neo⁺ colonies. Higher concentrations of thymidine also did not yield recombinants, but the toxicity of thymidine to HCT116 reduced the recovery of viable cells to a point in which meaningful measurements of the recombination frequency could not be made.

We next determined whether the thymidine-sensitive transfectants expressing the mutant Mre11 were deficient in the homology-directed repair of a site-specific DSB induced

in the recombination reporter construct. The Sneo recombination reporter has a site for the I-SceI endonuclease in one of the defective neo genes. This makes it possible to introduce a DSB into one of the defective neo genes by transfection of an I-SceI expression construct into the cells and to determine the frequency of its repair by a homology based recombination pathway (Johnson *et al.*, 1999). Two days after transfection of I-SceI, treated cells were plated on G418 to determine the frequency of neo⁺ recombinants. SW480/SN3, SM1.3, and SM1.6 cells showed an 87- to a 110-fold increase in the frequency of neo⁺ colonies (Figure 7B).

DISCUSSION

Here, we identify a mutant allele of Mre11 isolated from the MMR-defective colon cancer cell line HCT116 that confers sensitivity to thymidine and CPT in a dominant-negative manner. The frameshift in the T11 run in intron 4 of *MRE11* present in HCT116 (Giannini *et al.*, 2002) seems to generate a number of splice variants. The variant reported here results

Figure 6. Autophosphorylation of ATM after thymidine treatment is suppressed in cells expressing Δ_{5-7} Mre11 but not phosphorylation of Nbs1 or Chk1. (A and B) ATM autophosphorylation at Ser1981 was determined by Western blotting by using extracts of SW480/SN3, SM1.3, SM1.6, or HCT116 cells at the indicated times after exposure to 2 Gy of IR (A) or 0.5 mM thymidine (B). Levels of total ATM in these cells after treatment are also presented. (C) Autophosphorylation of ATM in parental 293 and FD40 cells after treatment with 0.5 or 10 mM thymidine for the indicated times. The levels of Chk2, pThr68-Chk2, Nbs1, pSer343-Nbs1, Chk1, and pSer345-Chk1 in extracts of 293 and FD40 cells treated with 10 mM thymidine for the indicated times was also analyzed by Western blotting. (D) Western blot analysis of Chk1, pSer345-Chk1, Nbs1, and pSer343-Nbs1 in extracts of 293 and FD40 treated with the indicated concentrations of thymidine for 1 h. The β -actin levels in B and C are presented as loading controls.

in the loss of exons 5-7, and it produces an in-frame transcript that encodes a 593-amino acid protein partially lacking a

highly conserved domain of Mre11. This region encodes the third and fourth of the five conserved phosphoesterase motifs

Figure 7. Cells expressing Δ_{5-7} Mre11 allele are defective in HR induced by thymidine, but they remain proficient in HR induced by a site-specific DSB. (A) Frequency of Scneo recombinants in SW480/SN3, SM1.3, SM1.6, or HCT116 after thymidine treatment. Six replica cultures were treated for each experiment, and the results shown are an average of two to four independent experiments. Recombination frequencies have been normalized to those obtained in untreated replica cultures. The background frequencies of neo+ recombinants were 7.2×10^{-7} for SW480/SN3, 1.1×10^{-6} for SM1.3, 1.1×10^{-6} for SM1.6, and 1.3×10^{-7} for HCT116/HN5. (B) Frequency of Scneo recombinants in SW480/SN3, strains expressing Δ_{5-7} Mre11 (SM1.3 and 1.6), and HCT116 after transfection of an expression construct for I-SceI. Results are an average of two to three independent experiments and error bars indicate standard deviations.

that are necessary for nuclease activity (Bressan *et al.*, 1998). The intact amino-terminal region of Mre11 is required for interaction with human Nbs1 (Desai-Mehta *et al.*, 2001), and it is predicted to be essential for interaction with Rad50 in *Pyrococcus furiosus* (Hopfner *et al.*, 2001; Williams *et al.*, 2007), although these interactions may be with homodimers of Mre11 that form through this domain (Hopfner *et al.*, 2001; D'Amours and Jackson, 2002). Consistent with this model, Δ_{5-7} Mre11 coimmunoprecipitates very poorly with Nbs1 and wild-type Mre11, and its interaction with Rad50 is impaired. Despite the apparent inability of the mutant Mre11 to form a functional MRN complex, it is still detected in the chromatin fraction of cells expressing the protein, particularly after replication fork stress induced by thymidine or CPT. Interestingly, a mutant allele of *MRE11* found in ATLD patients contains an amino acid substitution (N117S) in the same region that alters interactions with the remaining components of the MRN complex (Stewart *et al.*, 1999). In contrast, another mutant allele containing H129D and L130V substitutions in this region (hMre11-3) retains binding to DNA, Rad50, and Nbs1; however, nuclease activity was lost (Arthur *et al.*, 2004).

Not surprisingly Δ_{5-7} Mre11 is deficient in the 3'-5' exonuclease activity characteristic of the wild-type protein; however, it retains affinity for fork-like structures containing ssDNA regions. Replication intermediates containing such ssDNA regions could potentially form after dCTP depletion in thymidine-treated cells. Replication is likely to be retarded at G-rich regions on either lagging or leading strands when dCTP is limiting. In bacteria and human cell-free systems, lagging strand lesions may be bypassed by initiation of a new Okazaki fragment downstream from the damage leaving a small gap (Svoboda and Vos, 1995; Heller and Marians, 2006). Alternatively such forks may regress to form a chicken foot structure that presents a 3' ssDNA tail that can potentially bind the mutant Mre11 (Helleday, 2003). Leading strand lesions may cause uncoupling of leading and lagging strand synthesis leaving extended ssDNA stretches on the leading strand adjacent to fork junctions. Normally the wild-type Mre11 in association with PCNA can be found at ssDNA regions formed in response to replication fork arrest (Mirzoeva and Petrini, 2003). The accumulation of the exonuclease defective mutant Mre11 at such sites could prevent processing to intermediates that are resolved by HR or trigger ATM autophosphorylation (Figure 8). The parallel accumulation of hyperphosphorylated RPA and Δ_{5-7} Mre11 in the chromatin of mutant cells treated with thymidine supports the idea that unrepaired ssDNA sites persist in cells expressing the mutant protein. Furthermore the persistence of only a few lesions among the thousands of replication forks used during replication may be sufficient to compromise completion of S phase. Given the sensitivity of cells expressing Δ_{5-7} Mre11 to CPT, our results raise the possibility that this mutant protein may also interfere with the resolution of the collapsed replication forks formed by the collision of a fork with a topoisomerase I cleavage complex (Pommier, 2006). The 3' ssDNA-tailed molecule thought to be formed during the resolution of collapsed forks (Helleday, 2003) is recognized by the mutant Mre11 as indicated by our *in vitro* binding assays and the *in vivo* accumulation of the mutant protein in the chromatin of cells treated with CPT. This interference may not be complete as cells expressing the mutant Mre11 are able to repair single site-specific double-strand breaks induced in a recombination reporter substrate by HR.

Previous work suggests that the MRN complex functions as a sensor for DSBs (Petrini and Stracker, 2003) as a result of the ability of this complex to process such damage to a

Figure 8. Model for the effects of Δ_{5-7} Mre11 on the cellular response to replication inhibitors. Replication forks stressed by thymidine treatment trigger ATM and ATR signaling cascades that are necessary for repair and replication restart. Our evidence suggests that Δ_{5-7} Mre11 suppresses the autophosphorylation of ATM and HR-mediated restart of affected replication forks. The MRN complex also plays essential upstream and downstream roles in the cellular response DSBs induced by IR. Δ_{5-7} Mre11 does not seem to affect the activation of ATM in response to IR, and it may only weakly affect repair of DSBs as site specific DSBs induced in recombination reporter substrates are repaired in cells expressing the mutant Mre11.

form that activates checkpoints (D'Amours and Jackson, 2002). Hypomorphic mutations of Mre11 found in ATLD patients or defects in Nbs1 suppress ATM autophosphorylation at early times after induction of low levels of DSBs (Uziel *et al.*, 2003; Horejsi *et al.*, 2004). Our data support a similar role for the Mre11 complex in the ATM mediated response to thymidine-induced lesions (Figure 8). However the effect of Δ_{5-7} Mre11 is more robust, suppressing ATM activation even at later times (24 h after treatment) and high doses of thymidine. This might be the result of the retention of the mutant Mre11 at stalled DNA replication forks generated by thymidine treatment blocking processing to a form that recruits and activates ATM. However, the mutant Mre11 does not seem to suppress the ATR-Chk1 signaling that is triggered after thymidine treatment. Furthermore, phosphorylation of downstream ATM targets Nbs1 and Chk2 is evident, although less robust in cells expressing the mutant Mre11. Although this seems inconsistent with previous work showing that autophosphorylation of ATM at S1981 was essential for activation of these downstream targets (Bakkenist and Kastan, 2003), more recent work has shown that phosphorylation of these proteins after IR and ATM recruitment to sites of damage were not affected in mouse cells expressing a nonphosphorylatable ATM as their sole ATM species (Pellegrini *et al.*, 2006).

The role of Mre11 in HR has been the subject of some discussion (D'Amours and Jackson, 2002). In human cells, Rad51 and Mre11 foci that form after IR are generally mutually exclusive; however, it could not be ruled out that there might be a specific time point where the proteins cooperate (van Veelen *et al.*, 2005). In contrast, studies with *Xenopus* egg extracts have shown that Mre11 is required for the restart of collapsed replication forks and that ATM and ATR induce MRN complex redistribution to restarting forks (Trenz *et al.*, 2006). We previously reported that AT cells are also defective in HR induced by thymidine (Bolderson *et al.*, 2004). This may reflect a role for ATM and Mre11 in the HR-mediated restart of replication forks stalled by thymi-

dine treatment that is suppressed by the mutant Mre11 (Figure 8). Alternatively, the mutant Mre11 may bind to a substrate and block processing into an intermediate that is resolved by HR. As pointed out previously, the mutant Mre11 may reduce the efficiency of HR but not suppress it completely, because cells expressing Δ_{5-7} Mre11 are still able to repair single site-specific DSBs induced in an HR reporter construct. This may account for the sensitivity of cells expressing the mutant Mre11 to high doses of IR but not lower doses, because the residual level of HR may be able to cope with breaks induced by low levels of IR but not those at high doses.

The frameshift in the T11 run in intron 4 of *MRE11* that is likely to give rise to Δ_{5-7} Mre11 occurs in >80% of microsatellite instability+ colon cancers (Giannini *et al.*, 2002). Such frameshifts are hot spots for mutation in MMR-deficient tumor cells, suggesting that the Mre11 mutation occurs as a downstream event, after the loss of MMR, not unlike the mutations in *TGF β RII* or *BAX* genes (Markowitz *et al.*, 1995; Rampino *et al.*, 1997). Although it is not yet clear how loss of a damage response pathway to replication stress contributes to the development of this subset of tumors, it is interesting to note that hypoxia slows DNA replication as an apparent response to a decrease in dNTP supply caused by lower ribonucleotide reductase activity after oxygen depletion (Chimply *et al.*, 2000). This slowdown of DNA replication triggers p53 and targets hypoxic cells to apoptosis (Hammond *et al.*, 2002). We speculate that the mutant Mre11 gene may enable tumor cells arrested in S phase as a result of hypoxia to survive by interfering with the induction of apoptosis that requires a functional MRN complex (Stracker *et al.*, 2007). A potentially useful consequence of the loss of this pathway is the sensitization of affected cells to agents such as CPT that induce DSBs at replication forks. Work is underway to exploit the altered sensitivity conferred by this Mre11 mutation to develop novel therapies specifically targeted to tumors carrying this mutation.

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