**MRE11 Function in Response to Topoisomerase Poisons Is Independent of its Function in Double-Strand Break Repair in Saccharomyces cerevisiae**

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**Abstract**

Camptothecin (CPT) and etoposide (ETP) trap topoisomerase-DNA covalent intermediates, resulting in formation of DNA damage that can be cytotoxic if unrepaired. CPT and ETP are prototypes for molecules widely used in chemotherapy of cancer, so defining the mechanisms for repair of damage induced by treatment with these compounds is of great interest. In S. cerevisiae, deficiency in MRE11, which encodes a highly conserved factor, greatly enhances sensitivity to treatment with CPT or ETP. This has been thought to reflect the importance of double-strand break (DSB) repair pathways in the response to these agents. Here we report that an S. cerevisiae strain expressing the mre11-H59A allele, mutant at a conserved active site histidine, is sensitive to hydroxyurea and also to ionizing radiation, which induces DSBS, but not to CPT or ETP. We show that TDP1, which encodes a tyrosyl-DNA phosphodiesterase activity able to release both 5’- and 3’-covalent topoisomerase-DNA complexes in vitro, contributes to ETP-resistance but not CPT-resistance in the mre11-H59A background. We further show that CPT- and ETP-resistance mediated by MRE11 is independent of SAE2, and thus independent of the coordinated functions of MRE11 and SAE2 in homology-directed repair and removal of Spo11 from DNA ends in meiosis. These results identify a function for MRE11 in the response to topoisomerase poisons that is distinct from its functions in DSB repair or meiotic DNA processing. They also establish that cellular proficiency in repair of DSBS may not correlate with resistance to topoisomerase poisons, a finding with potential implications for stratification of tumors with specific DNA repair deficiencies for treatment with these compounds.

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**Introduction**

Topoisomerase poisons are potent drugs for treatment of cancer. Two naturally occurring topoisomerase inhibitors, camptothecin (CPT) and etoposide (ETP), are prototypes for this class of chemotherapeutics, which target Topoisomerase I (Topo I) and Topoisomerase II (Topo II) respectively. Topo I regulates DNA superhelicity ahead of transcription and replication forks by inserting itself into one strand of the DNA backbone, forming a 3’-covalent bond between a tyrosine residue on the enzyme and the nicked DNA strand and enabling rotation of the intact strand around the nick. CPT binds at the single-strand break 3’ of the Topo I-DNA complex, stabilizing the covalent Topo I-DNA intermediate and inhibiting religation of the DNA nick. Topo II promotes decatenation of DNA following replication, inserting into both strands of the duplex to form a double-strand break (DSB), with 5’-DNA ends tethered to tyrosine residues on Topo II subunits, enabling intact DNA to pass through the DSB. ETP intercalates at Topo II insertion sites, stabilizing the DSB intermediate. Defining the mechanisms of repair of damage induced by CPT and ETP is of considerable practical importance, because both are potent cytotoxic agents and their derivatives are commonly used in cancer chemotherapy.

Mutants of S. cerevisiae deficient in MRE11 (mre11A) are very sensitive to CPT [1,2,3] and to ETP [4]. MRE11 encodes a multifunctional nuclease, active as a 3’-5’ dsDNA exonuclease, a single-strand DNA endonuclease and an AP lyase in vitro, and shown to function in DNA repair, meiotic recombination, telomere maintenance and immunoglobulin gene diversification [5,6,7,8,9,10,11]. Its mechanism of function in response to CPT or ETP is not understood, but has been thought to correlate with activity in DSB repair, which is dependent upon its 3’-5’ exonuclease activity.

Here we describe a new S. cerevisiae mutant allele, mre11-H59A. We show that an S. cerevisiae mre11-H59A strain is as sensitive to CPT and IR as the well-characterized DSB repair-deficient mre11-H125N strain [3,10,12,13]. However, in contrast to the mre11-H125N strain, the mre11-H59A strain is resistant to CPT. CPT-resistance does not depend upon TDP1, which encodes a factor that releases covalent 3’- or 5’-tyrosyl DNA bonds in vitro [14,15,16,17]. The mre11-H59A and mre11-H125N strains are both resistant to ETP, which is toxic to the mre11A strain; but deficiency in TDP1 (tdp1Δ) causes these strains to become ETP-sensitive. Neither CPT- nor ETP-resistance of the mre11-H59A strain depends upon SAE2, which is required for MRE11-dependent removal of Spo11 from the ends of meiotic DNA [18] and regulates functions of MRE11 in homologous recombi-
nation [19]. Thus MRE11 has distinct functions in repair of damage induced by topoisomerase poisons and repair of mitotic and meiotic DSBs.

Results

*S. cerevisiae mre11-H59A* is Sensitive to Hydroxyurea but Not CPT

To distinguish functions of MRE11 in DSB repair and response to DNA damage by topoisomerase poisons, two previous findings drew our attention to the conserved active site histidine at residue 59 (H59) in phosphodiesterase motif II of *S. cerevisiae* Mre11 (Fig. 1A). Biochemical analysis had shown that purified recombinant *Pyrococcus furiosus* Mre11 with a mutation at this site (H52S) is deficient in exonuclease activity; while genetic analysis had shown that mutation at the corresponding position of the *Schizosaccharomyces pombe* Mre11 homologue (rad2-H68S) did not render cells sensitive to CPT, HU or IR [8].

In order to study the effect of mutation of H59 in *S. cerevisiae* and compare this allele to well-characterized mutant alleles, we generated a panel of strains bearing mutations in conserved residues in the Mre11 active site phosphodiesterase motifs (Fig. 1A). *S. cerevisiae* Jel1 mre11A (LSY1706, MATa leu2 trp1 ade2-1 his3-11,15 leu2-3,1122 pep4-5 his3-GAL10-GAL4 mre11a::HIS3MX6; [12]) was stably transformed with pYES2-2 μ vectors expressing C-terminal TAP-tagged Mre11 or mutant Mre11-H59A, Mre11-D56A, Mre11-D56N, Mre11-H125N or Mre11-H213Y under control of the GALI promoter, and gene and protein expression were confirmed by RT-PCR (not shown) and western blotting (Fig. S1). Mutations at D56 alter a conserved glutamate in close proximity to H59 in phosphodiesterase motif II [14]. However, *S. cerevisiae* tdp1A mutants exhibit only minor increases in sensitivity to CPT, either in strains expressing MRE11 or in the MRE11-deficient strains tested thus far, including mre11-H125N [1,3,17,25]. To compare function of TDP1 and MRE11 in CPT resistance, we created Jel1 tdp1A and Jel1 mre11A tdp1A derivatives, and transformed the latter with 2 μ plasmids expressing MRE11, mre11-H59A, mre11-H125N and mre11-H213Y mutant alleles. In spot dilution assays, the Jel1 mre11A strain was much more severely CPT-sensitive than the Jel1 tdp1A strain, and sensitivity was not enhanced in the Jel1 mre11A tdp1A double mutant (Fig. 3). Moreover, the Jel1 tdp1A mre11A derivatives expressing MRE11, mre11-H59A, mre11-H125N or mre11-H213Y exhibited CPT-sensitivity essentially indistinguishable from the corresponding Jel1 mre11A derivatives (Fig. 3, compare lower and upper). Thus, MRE11 was more critical to CPT-resistance than TDP1, and deficiency in TDP1 did not affect CPT sensitivity of MRE11-deficient strains.

**TDP1 Does Not Contribute to CPT-Resistance of MRE11-Deficient Strains**

The enzyme Tyrosyl-DNA-phosphodiesterase 1 (Tdp1) was identified as an activity in *S. cerevisiae* extracts capable of removing a trapped Topo I-DNA complex in vitro [14]. However, *S. cerevisiae* tdp1A mutants exhibit only minor increases in sensitivity to CPT, both in strains expressing MRE11 or in the MRE11-deficient strains tested thus far, including mre11-H125N [1,3,17,25]. To compare function of TDP1 and MRE11 in CPT resistance, we created Jel1 tdp1A and Jel1 mre11A tdp1A derivatives, and transformed the latter with 2 μ plasmids expressing MRE11, mre11-H59A, mre11-H125N and mre11-H213Y mutant alleles. In spot dilution assays, the Jel1 mre11A strain was much more severely CPT-sensitive than the Jel1 tdp1A strain, and sensitivity was not enhanced in the Jel1 mre11A tdp1A double mutant (Fig. 3). Moreover, the Jel1 tdp1A mre11A derivatives expressing MRE11, mre11-H59A, mre11-H125N or mre11-H213Y exhibited CPT-sensitivity essentially indistinguishable from the corresponding Jel1 mre11A derivatives (Fig. 3, compare lower and upper). Thus, MRE11 was more critical to CPT-resistance than TDP1, and deficiency in TDP1 did not affect CPT sensitivity of MRE11-deficient strains.

**TDP1 Contributes to ETP-Resistance of mre11-H59A and mre11-H125N Mutants**

In *S. cerevisiae*, MRE11-deficiency causes sensitivity to ETP, which traps covalent complexes formed by Topo II with DNA 5′-ends [4]. We therefore tested ETP-sensitivity of a panel of MRE11-deficient strains in a spot dilution assay. We found that the Jel1 mre11A strain and its derivative expressing mre11-H213Y were extremely sensitive to ETP; the derivatives expressing mre11-H59A and mre11-H125N were no more sensitive than the derivative expressing MRE11; and the derivatives expressing mre11-D56A and mre11-D56N were relatively resistant (Fig. 1B, left). Spot tests also showed that Jel1 mre11A and its derivative expressing mre11-H213Y were extremely sensitive to CPT, the derivatives expressing mre11-D56A, mre11-D56N and mre11-H125N slightly less sensitive, and the derivative expressing mre11-H59A relatively resistant, although slightly less so than the derivative expressing MRE11 (Fig. 1B, right). Identical results were obtained using the low copy number CEN plasmid p46ADH expressing MRE11, mre11-H59A or mre11-H213Y alleles (data not shown). Thus, the mre11-H59A mutation caused sensitivity to HU but not to CPT, as measured by spot dilution assays.

The contrasting effects of the mre11-H59A mutation on HU and CPT sensitivity were confirmed by clonogenic survival assays. These assays showed that the strain expressing mre11-H59A was sensitive to HU, less so than the extremely HU-sensitive Jel1 mre11A strain or its derivative expressing mre11-H213Y, but more sensitive than the parental Jel1 strain or the Jel1 mre11A derivatives expressing MRE11, mre11-D56A, mre11-D56N or mre11-H125N (Fig. 1C, left). Nonetheless, the Jel1 mre11A derivative expressing mre11-H59A was as resistant to CPT as the Jel1 parental line or the Jel1 mre11A derivative expressing MRE11 (Fig. 1C, right).
Figure 1. The mre11-H59A allele confers sensitivity to HU but not CPT. A. Conserved phosphodiesterase motifs in the active site of the Mre11 protein. Sequences of conserved amino acid residues in Motifs I–V are shown for three different organisms: S. cerevisiae (Sc), S. pombe (Sp), and Homo sapiens (Hs). Effects of mutations at S. cerevisiae residues shown in red in were tested in experiments described herein. The lengths of the Mre11 polypeptides are all different, and positions of the conserved residues in each motif are shown below for reference: Sc, I, H18; II, D56, H59; III, H125; IV, H213; V, H241, H243; Sp, I, H13; II, D65, H68; III, H134; IV, H222; V, H250, H252; Hs, I, H22; II, D60, H63; III, H129; IV, H217; V, H245, H247. B. Serial spot dilution assays of sensitivity to HU and CPT of S. cerevisiae wild-type parental line Jel1 (Jel1); or its MRE11-deficient derivative, Jel1 mre11D, stably transformed with 2 µ plasmid vectors expressing protein as indicated, including empty vector (Jel1 mre11D + 2µ), wild-type MRE11 (+2 µ MRE11), or indicated mutant alleles (+2 µ mre11-D56A, +2 µ mre11-D56N, +2 µ mre11-H59A, +2 µ mre11-H125N, +2 µ mre11-H213Y). Cells were spotted at 10-fold serial dilutions (indicated by triangles) on rich plates containing no drug (left) or indicated concentrations of HU or CPT. C. Clonogenic survival assays of sensitivity to HU and CPT. Strains were assayed for colony formation at indicated doses of each compound. Survival was normalized to untreated samples; error bars indicate standard error of the mean.

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caused much more severe ETP-sensitivity than deficiency in TDP1. Moreover, while deficiency in TDP1 did not affect ETP-sensitivity of the Jel1 mre11D derivative expressing MRE11, it did cause a modest increase in ETP-sensitivity of the Jel1 mre11D derivative expressing mre11-H59A, and a more severe increase in sensitivity of the derivative expressing mre11-H125N (Fig. 4, lower).

**SAE2 Makes a Modest Contribution to CPT- or ETP-Resistance**

SAE2 encodes a repair endonuclease that is essential for Mre11 function in removal of Spo11 from 5’-ends of DNA in meiosis, and that regulates activities of Mre11 in homologous recombination [18,19,26]. Deficiency in SAE2 has been reported not to affect CPT- or ETP-sensitivity in *S. cerevisiae* [3,18], although the SAE2 ortholog, CIP, is essential for CPT-resistance in *S. pombe* [27]. Because of the role of SAE2 in meiosis, it seemed important to test the effect of deficiency in SAE2 on CPT- and ETP-sensitivity of the Jel1 mre11D derivative expressing mre11-H59A. We therefore generated Jel1 sae2Δ and Jel1 sae2Δ mre11A derivatives, and Jel1 sae2Δ mre11A derivatives expressing MRE11, mre11-H59A or mre11-H213Y alleles (the latter was included as a control due to its severe meiotic defect). Consistent with the role of SAE2 in DSB repair, strains deficient in SAE2, including Jel1 sae2Δ, Jel1 sae2Δ mre11A, and derivatives of the latter expressing MRE11, mre11-H59A, or mre11-H213Y, all exhibited greater HU sensitivity than the parental SAE2 strains (Fig. 5A). However, deficiency in SAE2 caused only a very modest increase in CPT-sensitivity (Fig. 5B) or ETP-sensitivity (Fig. 5C). Thus, SAE2 is not critical to MRE11-dependent repair of damage induced by CPT or ETP.

**Discussion**

We have shown that the *S. cerevisiae* mre11-H59A strain is resistant to topoisomerase poisons CPT and ETP, which create protein-DNA covalent complexes; but sensitive to HU and sensitive to IR, which induces DSBs. These results establish that the MRE11 function in DSB repair can be separated from its function in repair of CPT- or ETP-induced DNA damage. They also establish that a cell need not be proficient in repair of DSBs to resist topoisomerase poisons.

Our results identify several clear distinctions between *S. cerevisiae* and *S. pombe* in the response to topoisomerase poisons. One

![Figure 2](https://example.com/figure2.png)

Figure 2. The *mre11-H59A* allele confers sensitivity to IR. Cell survival was measured following indicated exposure to radioactive ¹³⁷Cs, and normalized to unirradiated samples. Error bars indicate standard error of the mean. Strain notations as in Fig. 1. doi:10.1371/journal.pone.0015387.g002

![Figure 3](https://example.com/figure3.png)

Figure 3. *TDP1* does not contribute to CPT-resistance of *MRE11*-deficient strains. Serial spot dilution assays of sensitivity to CPT of *S. cerevisiae* Jel1 TDP1 or Jel1 TDP1 mre11D (above), and Jel1 tdp1Δ or Jel1 tdp1Δ mre11D (below). The mre11D derivatives were stably transformed with 2 µ plasmid vectors, including empty vector (Jel1 mre11D +2 µ) or vectors expressing wild-type MRE11 (Jel1 mre11D +2 µ MRE11) or indicated mutant alleles (+2 µ mre11-H59A, +2 µ mre11-H125N, +2 µ mre11-H213Y). Cells were spotted at 10-fold serial dilutions on rich plates containing no drug (left) or indicated concentrations of CPT. doi:10.1371/journal.pone.0015387.g003
difference is in functions associated with specific residues of the highly conserved active site of the Mre11 polypeptide. The *S. cerevisiae* mre11-H59A strain that we have studied carries a mutation at a conserved active site histidine in phosphodiesterase motif II of the active site and is resistant to CPT but sensitive to HU and IR. An *S. pombe* strain carrying a mutation at the corresponding position, rad32(mre11)-H68S, is resistant to CPT and also to HU and IR [8]. The repair pathways for CPT- and ETP-induced damage in *S. cerevisiae* and *S. pombe* also exhibit distinct dependence upon other factors. In *S. cerevisiae*, TDP1-deficiency has little effect on CPT-sensitivity, either in strains expressing wild-type MRE11 [1,25] or in the four mre11 mutant strains we examined. This contrasts with *S. pombe*, where TDP1-deficiency has a pronounced effect on CPT-sensitivity [28]. In *S. cerevisiae*, SAE2 makes only a very modest contribution to CPT- or ETP-resistance. This also contrasts with *S. pombe*, where deficiency in the SAE2 ortholog, CtIP, impairs the ETP response, but surprisingly promotes the CPT response [27].

How might MRE11 function to prevent toxicity by topoisomerase poisons independent of DSB repair? A critical step that distinguishes the response to topoisomerase poisons from other cytotoxic treatments is removal of the covalent protein-DNA complexes that accumulate in cells treated with these compounds. Our results raise the possibility that MRE11 may promote cleavage of tyrosyl-DNA covalent bonds, either directly or indirectly. Several factors possess this activity, including the conserved enzyme Tdp1, which can cleave both 3' and 5' covalent tyrosyl-DNA bonds in vitro [14,15,16,17]; the structure-specific nucleases Rad1/Rad10 and Mms4/Mus81, which appear to function redundantly with Tdp1 in release of 3'-covalent protein DNA complexes in vivo [29]; and the recently discovered human factor, Tdp2, which can cleave 5’-tyrosyl-DNA covalent bonds in vitro and rescue CPT-sensitivity of *S. cerevisiae* tdp1A rad1A mutants in vivo [30]. Consistent with a role for MRE11 in promoting cleavage of tyrosyl-DNA covalent bonds, topoisomerase-DNA complexes have been shown to persist in an *S. pombe* rad32(mre11)-D65N strain following treatment with CPT or ETP [27].

Determining the mechanism of MRE11 function in the response to topoisomerase poisons has important implications for the clinical setting. A subset of human colorectal cancers is MRE11-deficient [31,32]. If MRE11 functions to promote release of covalent topoisomerase-DNA complexes independent of its role in DSB repair, MRE11-deficient tumors may be priority candidates for treatment with CPT and ETP derivatives. This could have implications for stratification of tumors with specific DNA repair deficiencies for treatment with topoisomerase poisons.

### Materials and Methods

**S. cerevisiae Strains**

Strains were derived from Jel1 (*Mat*α *ura3-52 pep4-3 his3:3-jpGAL10-GAL4* or *Jel1 mre11A* (*Mat*α *ura3-52 pep4-3 his3:3-jpGAL10-GAL4 mre11::HIS3*), kindly provided by Dr. Lorraine Symington, Columbia University [12]). The tdp1A and sae2A derivatives were constructed using the PCR method of gene deletion [33], which replaces the target gene by a G418 resistance marker. G418-resistance markers were amplified from *S. cerevisiae* tdp1D and sae2D strains, kindly provided by Dr. Stanley Fields (University of Washington). Primers, designed to provide homology arms of about 500 bp, were:

- **Sae2 F**: 5’-CAGTAATTTGACGATGCGGAAGG
- **Sae2 R**: 5’-CGACGTTCTCTATCATAATAAAACCCTGG
- **Tdp1 F**: 5’-CAGCATTTTTATGTTCAGTAATCATT-GAACTTG
- **Tdp1 R**: 5’-GGAGCATCTATTAAAAAGAGCTTTTAATC

[Figure 4. TDP1 contributes to ETP-resistance of mre11-H59A and mre11-H125N strains. Serial spot dilution assays of sensitivity to ETP of indicated *S. cerevisiae* derivatives of parental line Jel1; notations as in Fig. 3. Cells were spotted at 10-fold serial dilutions on rich plates containing no drug (left) or indicated concentrations of ETP. doi:10.1371/journal.pone.0015387.g004]
PCR products were purified and transformed into Jel1 or mre11A strains using the lithium acetate method. Integrants were selected on either CSM-URA or YPD plates containing 200 μg/ml G418, and deletion verified by sequencing the products of colony PCR.

**MRE11 Expression Constructs**

*MRE11* was PCR-amplified from Jel1, using PCR primers 5'-GGGGTACCATGGTCATCATCAC and 5'-CTAGTCTAGATTCTTCTTTTTTATTATGAGGAGACGTTCAGGATCAGCTTCGCCGCTTTCCGGTCCGAG to create an XbaI site and consensus initiation sequence at the 5' end and remove the stop codon and create an XbaI site at the 3' end (KpnI and XbaI sites underlined). DNA was digested with KpnI and XbaI and inserted into the KpnI and XbaI sites of a 2 μ plasmid carrying the URA3 selectable marker (Invitrogen, Carlsbad, CA). A TAP tag was amplified from a construct provided by Dr. Trisha Davis (University of Washington), using primers 5'-CTAGTCTAGTTCTTTTTTTTTATTATGAGGAGACGTTCAGGATCAGCTTCGCCGCTTTCCGGTCCGAG and 5'-CTAGTCTAGTTCTTTTTTTTTATTATGAGGAGACGTTCAGGATCAGCTTCGCCGCTTTCCGGTCCGAG to create XbaI sites (underlined) on both 5' and 3' ends, XbaI-digested and inserted into the XbaI-digested pYES2-*MRE11* construct, and screened for directional insertion, generating pYES2-*MRE11*-TAP. *MRE11* was expressed from the GAL1 promoter in these plasmids. Mutants were generated by QuikChange (Stratagene, La Jolla, CA) and verified by sequencing, using the following primers and their complements (mutations underlined): D56A: 5'-ATGGTTGTACATGGTCATCATCAC and 5'-CTAGTCTAGATTCTTCTTTTTTATTATGAGGAGACGTTCAGGATCAGCTTCGCCGCTTTCCGGTCCGAG; D56N: 5'-ATGGTTGTACATGGTCATCATCAC and 5'-CTAGTCTAGATTCTTCTTTTTTATTATGAGGAGACGTTCAGGATCAGCTTCGCCGCTTTCCGGTCCGAG; D56N, H125N: 5'-ATGGTTGTACATGGTCATCATCAC and 5'-CTAGTCTAGATTCTTCTTTTTTATTATGAGGAGACGTTCAGGATCAGCTTCGCCGCTTTCCGGTCCGAG; H125N: 5'-ATGGTTGTACATGGTCATCATCAC and 5'-CTAGTCTAGATTCTTCTTTTTTATTATGAGGAGACGTTCAGGATCAGCTTCGCCGCTTTCCGGTCCGAG; H213Y: 5'-ATGGTTGTACATGGTCATCATCAC and 5'-CTAGTCTAGATTCTTCTTTTTTATTATGAGGAGACGTTCAGGATCAGCTTCGCCGCTTTCCGGTCCGAG.

**Western Blotting**

Approximately 5 × 10⁶ cells were pelleted, resuspended in 500 μl lysis buffer (25 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.5% NP-40, 10% glycerol, 1 mM PMSF, 1 mM DTT, 150 mM NaCl, 1xRoche Complete Protease Inhibitor), then lysed by vortexing with 500 μl glass beads at full speed 5 × 1 min, inculating at least 1 min on ice between vortexing. Following SDS-PAGE electrophoresis and transfer, blots were probed using polyclonal anti-ScMre11 (GeneTex) at 1:2000 and anti-β-actin (Abcam) at 1:2000. The anti-β-actin was used to normalize the signal, as its expression levels vary less widely between the different strains.

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