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Trypanosoma brucei MRE11 is non-essential but influences growth, homologous recombination and DNA double-strand break repair☆

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Abstract

MRE11 is a conserved multi-functional protein that is important for maintaining genomic integrity in yeast and mammalian cells. By database searching, we identified a full-length candidate *MRE11* on *Trypanosoma brucei* chromosome II. We subsequently cloned and sequenced the corresponding gene from the Lister 427 strain. *MRE11* is a single copy gene that encodes an 83 kDa protein of 763 amino acids. GFP-MRE11 and Ty1-MRE11 fusion proteins localized to the nucleus of bloodstream and procyclic *T. brucei*. Interestingly, Ty1-MRE11 associated, to some extent, with telomeres of procyclic but not bloodstream forms. This association appears cell-cycle dependent, with the highest co-localization in G1 cells. We were able to generate an *MRE11* null mutant in bloodstream forms, indicating that it is non-essential. However, the null mutant was impaired in homologous recombination, as evidenced by the reduced integration efficiency of transfected DNA. A conditional null mutant, containing a tetracycline-inducible ectopic *Ty1-MRE11*, exhibited reduced growth and plating efficiency and increased sensitivity to DNA double-strand breaks, induced by methyl methanesulphonate or ionizing radiation, in the absence of tetracycline.

Keywords: *Trypanosoma brucei*; MRE11; Growth; DNA repair; Recombination; Telomere

1. Introduction

Discovered in yeast mutants defective in meiotic recombination [1], MRE11 is a multi-functional nuclear protein that is important for maintaining genomic integrity (reviewed in Refs. [2,3]). In yeast and mammalian cells, MRE11 forms a tripartite complex with RAD50 and Xrs2 or Nbs1, respectively. This complex has been implicated in multiple cellular functions, including telomere maintenance [4–9], DNA damage response [10–13], non-homologous end-joining [14–17], processing of DNA double-strand breaks (DSBs) during mitotic homologous recombination, and formation and processing of meiotic DSBs [18–21]. MRE11 is essential in vertebrates, precluding detailed functional analysis of the complex, although there has been a report on the analysis of MRE11 null chicken DT40 cells that had been generated conditionally [22]. MRE11 deficiency in these cells resulted in accumulation of chromosome breaks and radiosensitivity. In humans, mutations in MRE11 and NBS1 result in A-TLD (ataxia-telangiectasia-like disease) and NBS (Nijmegen breakage syndrome). Cells from A-TLD and NBS patients are also characterized by radiosensitivity and chromosomal fragility [12]. In yeast, MRE11 is important for meiotic recombination and ionizing radiation resistance [1]. Null mutants display a variety of phenotypes, including increased sensitivity to DNA damaging agents, poor mitotic growth, increased rates of spontaneous mitotic recombination between heteroalleles in diploids, shortened telomeres and inability to create meiotic DSBs (reviewed in Ref. [3]).

Trypanosoma brucei is a unicellular hemoflagellate that diverged very early in the eukaryotic lineage [23].

Abbreviations: DSB, DNA double-strand break; MMS, methyl methanesulphonate; VSG, variant surface glycoprotein.

☆ *Note:* Nucleotide sequence data reported in this paper are available in the GenBank™ database under the accession number AY077640.

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The parasite is enveloped by a dense surface coat consisting of a single member of the variant surface glycoprotein (VSG) family. It is the periodic switching of VSGs, a process known as antigenic variation, that allows these parasites to escape the host immune response (reviewed in Refs. [24,25]). While there has been considerable progress in understanding the molecular mechanisms involved in antigenic variation [26], relatively less is known about other aspects of *T. brucei* chromosome biology. Investigations on conserved genes are increasingly feasible since much of the *T. brucei* genome sequence became available for analysis. The existence of powerful molecular tools such as gene transfection, gene knockout and inducible expression systems [27,28], has facilitated the functional analysis of gene homologues.

There have been single reports describing the DNA damage repair and recombination protein RAD51 in *T. brucei* and *Leishmania major* [29,30]. However, nothing is known about other DNA repair and recombination proteins in *T. brucei*. In this paper, we report the identification and characterization of an MRE11 homologue in *T. brucei*. Our study suggests that the DNA damage repair pathway is highly conserved among the eukaryotes, even for the evolutionarily diverged *T. brucei*.

2. Materials and methods

2.1. Transgenic trypanosome cell lines and DNA constructs

Bloodstream-form *T. brucei* (strain Lister 427, antigenic type MITat 1.2, clone 221a [31]) was cultured in HMI-9 at 37 °C as previously described [32]. This strain had been previously manipulated to generate bloodstream and procyclic cell lines 'single-marker' and 29-13 [28]. Both cell lines constitutively express T7 RNA polymerase and Tet repressor. Bloodstream 'single-marker' forms were cultured in HMI-9 containing 2.5 μg ml^{-1} G418 (Sigma) to a maximum cell density of 2×10^6 cells ml^{-1}. Procyclic forms (29-13) were cultured in SDM-79 [33] containing 15 μg ml^{-1} G418 and 25 μg ml^{-1} hygromycin (Sigma). The gene encoding the Ty1 (GluValHisThrAsnGlnAspProLeuAsp)-MRE11 fusion was cloned into the *Bam*HI–*Hin*dIII digested plasmid pLew82 [27], replacing the resident luciferase gene. The GFP-MRE11 fusion was generated by first amplifying the *MRE11* ORF using primers flanked by *Bam*HI restriction sequences. The 2.3-kb PCR product was cloned into *Bam*HI-digested pCO55, a pLew82 derivative that introduces a GFP tag at the N-terminus of an inserted protein. These T7 promoter driven cassettes were integrated into the rDNA spacer of the 'single-marker' cell line. Expression was induced by adding 2.5 or 500 ng ml^{-1} of tetracycline to the medium when required.

The constructs used to generate *MRE11* deletion mutants contained ~300 bp of 5′ and 3′ *MRE11* coding sequence, for targeting, separated by drug resistance cassettes (Fig. 3). The hygromycin phosphotransferase ORF, flanked by ~100 bp of 5′ and 300 bp of 3′ splicing signals from the actin locus, was removed from pHD309 [34] by *Sma*I–*Stu*I digestion. This fragment was cloned into the *GFP-MRE11* construct that had been digested with *Nde*I and *Nsi*I and T4 DNA polymerase-treated, essentially replacing the internal region of *MRE11* with the drug-resistance cassette. The final construct was cut with *Bam*HI, to liberate the targeting cassette, prior to transfection of 'single-marker' cells. The puromycin *N*-acetyltransferase ORF, flanked by ~100 bp of 5′ and 220 of 3′ splicing signals from the actin locus, was cloned into the *GFP-MRE11* construct that had been digested with *Nde*I and *Nsi*I and T4 DNA polymerase-treated. The final construct was cut with *Sma*I and *Eco*NI, to liberate the targeting cassette, for transfection. All transfections were performed as previously described [34].

2.2. DNA analysis

Southern analysis was carried out according to standard protocols [35]. Approximately 2 μg of DNA was run on a 0.8% agarose gel and transferred to a nitrocellulose membrane. Probes were generated using a Prime-It II labeling kit (Stratagene) with gel-purified DNA. Hybridization and washing of membranes were carried out at 65 °C in a hybridization oven (Hybaid). Post-hybridization washes were for 30 min at 65 °C in 0.1 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% SDS. Hybridization signals were detected by autoradiography.

2.3. Western blotting

1×10^6 bloodstream forms were lyzed in 10 μl of loading buffer (50 mM Tris Cl pH 6.8, 5% 2-mercaptoethanol, 2% SDS, 0.1% bromophenol blue, 10% glycerol) by boiling for 7 min. The cell extracts were separated on a 10% SDS-PAGE gel, electroblotted onto nitrocellulose and blocked with 5% fat-free milk powder in PBS. Ty1-tagged MRE11 was detected by incubating blots with anti-Ty1 monoclonal antibody (BB2) [36]. Blots were washed, incubated with horseradish peroxidase-conjugated sheep anti-mouse antibody (Amersham-Pharmacia), washed again and developed in SuperSignal substrate (Pierce), according to the manufacturer's instructions.

2.4. In situ hybridization and immunofluorescence

This protocol was carried out as described previously [37], with minor modifications. Briefly, 1×10^6 bloodstream or procyclic forms, induced with tetracycline, were washed with trypanosome dilution buffer [38] or PBS, respectively, before fixing in 2% formaldehyde for 10 min at 4 °C. The fixed cells were attached onto glass coverslips by centrifugation at $2000 \times g$ for 5 min at 4 °C and permeabilized with 0.2% NP-40 in PBS for 10 min at room temperature. For in situ hybridization, DNA was denatured by incubating permeabilized cells in 70% formamide/$2 \times$ SSC for 5 min at 70 °C. The coverslips were washed briefly in $2 \times$ SSC and probed with a digoxigenin-labeled $(CCCUAA)_{27}$ RNA probe. Cells were blocked with PBG (PBS with 0.1% cold fish gelatin, Sigma, and 0.5% BSA) and incubated with sheep anti-digoxigenin (Boehringer). BB2 mouse monoclonal IgG (gift of Keith Gull) and anti-GFP rabbit polyclonal IgG (Molecular Probes) were used to detect Ty1- or GFP-tagged MRE11, respectively, and DAPI was used to stain DNA. BB2 and anti-GFP were visualized with fluorescein isothyocyanate-conjugated donkey anti-mouse or -rabbit antibodies (Jackson Laboratories), respectively. Cells were mounted in antifade mounting solution (Vectashield, Vecta Laboratories Inc.) and viewed with a fluorescence microscope (Nikon Optiphot). Representative images were captured using a CCD camera (Spot RT, Diagnostic Instruments Inc.), with pseudo coloring and overlays facilitated with Adobe Photoshop. Image acquisition for deconvolution microscopy was done using a DeltaVision image restoration microscopy system (Applied Precision). Images were acquired in 0.2 μm steps in the z-axis and deconvolution was performed using the softWoRx^{TM} V 3.0 Software.

2.5. Colony growth

Bloodstream forms were grown as colonies on agarose plates as described previously [39], with minor modifications. Briefly, 6.5 g of agarose (low melting temperature, type VII, Sigma A4018) were dissolved in 100 ml of water and autoclaved. The melted 6.5% agar solution and 200 ml of HMI-9 were equilibrated to 50 °C in a water bath. Ten parts of medium were mixed with one part of agarose and 22 ml aliquots were poured into sterile Petri dishes. Plates were allowed to set for 30 min at room temperature or overnight at 4 °C. Before use, plates were pre-dried in a sterile hood with lids off, for exactly 1 h. Cells were counted and resuspended to a density of 10^3 cells ml^{-1} in HMI-9. Five hundred microliters of cell suspension were dispensed onto the plates and evenly dispersed using a glass spreader. The plates were again dried, lids off, for another 10 min. The plates were incubated overnight in a 37 °C humid CO_2 incubator, to allow for pH equilibration. Each plate was subsequently sealed in Parafilm to prevent drying and further incubated for 4–5 days. Colonies became visible around day 5.

2.6. MMSand ionizing radiation assays

One hundred cells were resuspended in 15–20 ml of HMI-9. The suspension was exposed to varying doses of ionizing radiation (Gamma-Cell 1000 ^{137}Cs Irradiation Unit at a dose rate of approximately 800 rads min^{-1}) or different concentrations of MMS (Sigma). The treated cells and untreated controls were distributed into 96-well plates, in duplicate, and incubated overnight in a 37 °C humid CO_2 incubator to allow for pH equilibration. Each plate was subsequently sealed with Parafilm to prevent drying and positive wells were enumerated after further incubation for 6–7 days.

2.7. Recombination assay

Homologous recombination efficiently was measured by electroporating a G418-selectable tubulin-targeted construct (*Not*I-digested pLew 114 [28]) into equal numbers of wild-type and mutant cells, which were distributed onto 12-well plates and selected by adding 2.5 μg ml^{-1} of G418 after 24 h. Positive wells were counted after 5 more days.

3. Results

3.1. Cloning and characterization of the T. brucei MRE11 gene

Using tBLASTn, a full-length putative orthologue of *Saccharomyces cerevisiae* MRE11 was identified on a bacterial artificial chromosome clone derived form *T. brucei* strain 927 chromosome II (The Institute for Genome Research: GenBank accession number AC00865). The complete coding region of the gene was amplified from strain Lister 427 by PCR, cloned and sequenced (GenBank accession number AY077640). *MRE11* encodes a 763-amino acid, 83.4-kDa, polypeptide. Alignment of the predicted amino acid sequences of the conserved amino-terminal region of *T. brucei*, budding yeast, fission yeast, plant and human MRE11, is shown in Fig. 1. Over this region, *Tb*MRE11 shares 32% identity with *S. cerevisiae*, 35% identity with *Schizosaccharomyces pombe*, 34% identity with *Arabidopsis thaliana*, and 37% identity with human MRE11. Sequence conservation is most striking in the phosphoesterase motifs, which are known to be important for nuclease activity in yeast MRE11 [40].

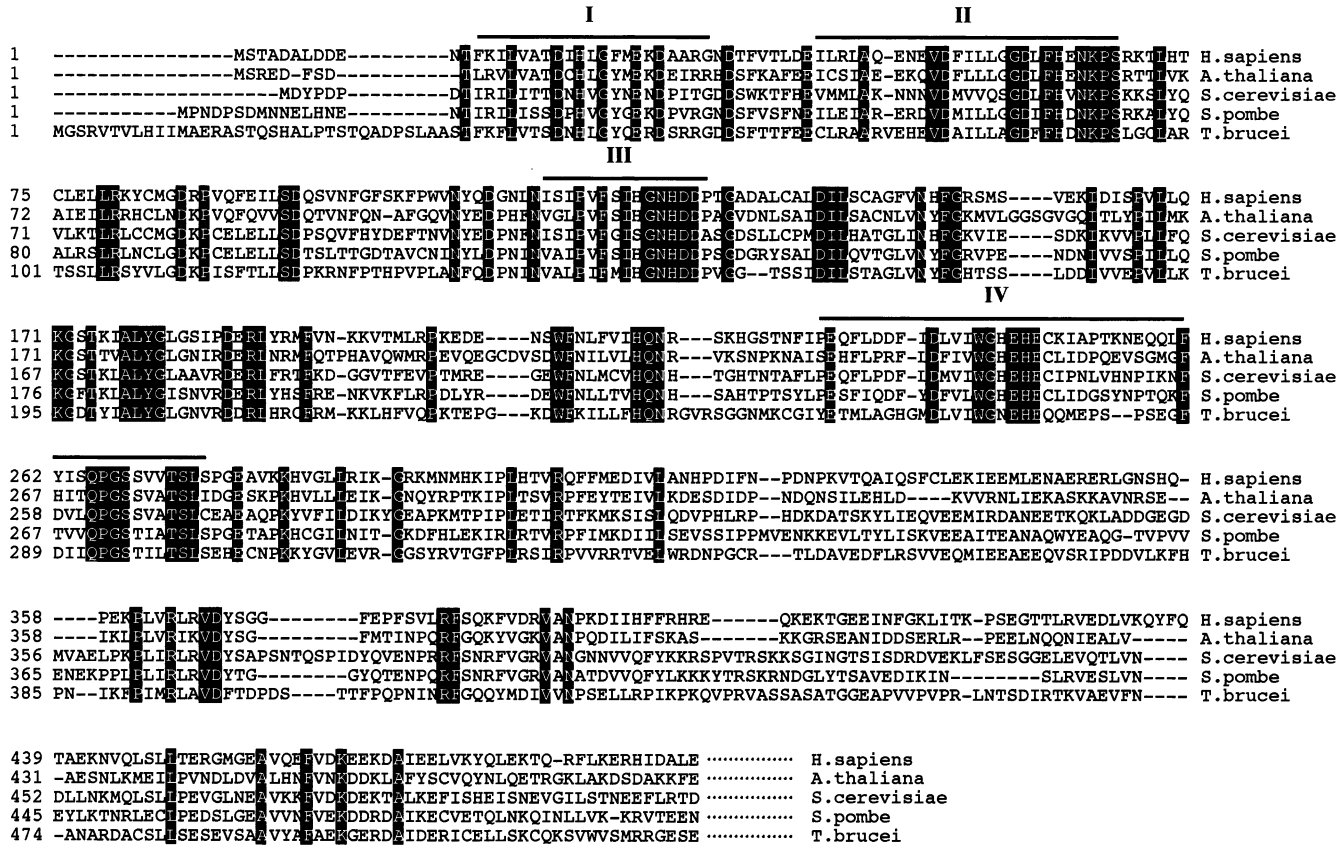

Fig. 1. Amino acid sequence alignment of the conserved amino-terminal region of selected MRE11 proteins. Deduced amino acid sequences were aligned using MegAlign (Lasergene/DNASTAR) according to the Clustal W method. Sequences used for comparison are Lister 427 *T. brucei* (GenBank accession number AY077640), *S. cerevisiae* (NC001145), *S. pombe* (Q09683), *Homo sapiens* (NM005590) and *A. thaliana* (NM124806). Residues that are identical in all the sequences are highlighted on a black background. Overlined regions indicate phosphoesterase motifs known to be important for nuclease activities in *S. cerevisiae*.

3.2. Expression and localization of MRE11 in T. brucei

By Southern and northern analysis, it was observed that MRE11 is a single-copy gene with similar steady state transcript levels in both bloodstream and procyclic forms (data not shown). Antibodies to human and *S. cerevisiae* MRE11 did not cross react with the *T. brucei* protein (western blot: data not shown). Insertion of tetracycline-inducible ectopic copies of GFP- and Ty1-tagged MRE11, into both bloodstream and procyclic forms, allowed us to study the localization of the protein while obviating the need for *Tb*MRE11 antibodies. GFP-MRE11 clearly localized to the nucleus, but its intrinsic fluorescence was barely visible by eye, despite high induction by tetracycline (results not shown), suggesting that the parasite was somehow limiting the expression of the ectopic protein. By immunofluorescence, the nuclear localization of Ty1- or GFP-tagged MRE11 was not uniform, but was manifested as small punctate spots in bloodstream forms and larger agglomerations in procyclic forms (Fig. 2). Interestingly, bloodstream forms in G2-M phases of the cell-cycle appeared to have higher levels of the protein, suggesting some form of cell-cycle regulation. Because MRE11 interacts with telomere-binding proteins in *S. cerevisiae* [8,9] and mammalian cells [6], and a small fraction can be visualized at telomeric sites in mammalian cells [6], we investigated whether the sub-nuclear pattern we observed was associated with telomeres. By fluorescence in situ hybridization, using a telomeric-repeat-specific

Fig. 2. MRE11 localizes to nucleus of *T. brucei* and partially colocalizes to telomeres of procyclic forms. (A) Deconvolution images of procyclic forms; (B) single-focal-plane images of bloodstream forms. In situ hybridization for TTAGGG repeats indicates where telomere DNA is located (red fluorescence) and indirect immunofluorescence assay shows distribution of *Tb*MRE11 (green fluorescence) within DAPI-stained nuclei (blue fluorescence). The left panels show representative trypanosomes in G1 with one kinetoplast (K) and one nucleus (N). The middle panels show G2-M phase 2K1N trypanosomes, and the right panels depict post-mitotic 2K2N trypanosomes. There is some accumulation of MRE11 around the nucleolus and enrichment near or at some G1 procyclic-form telomeres.

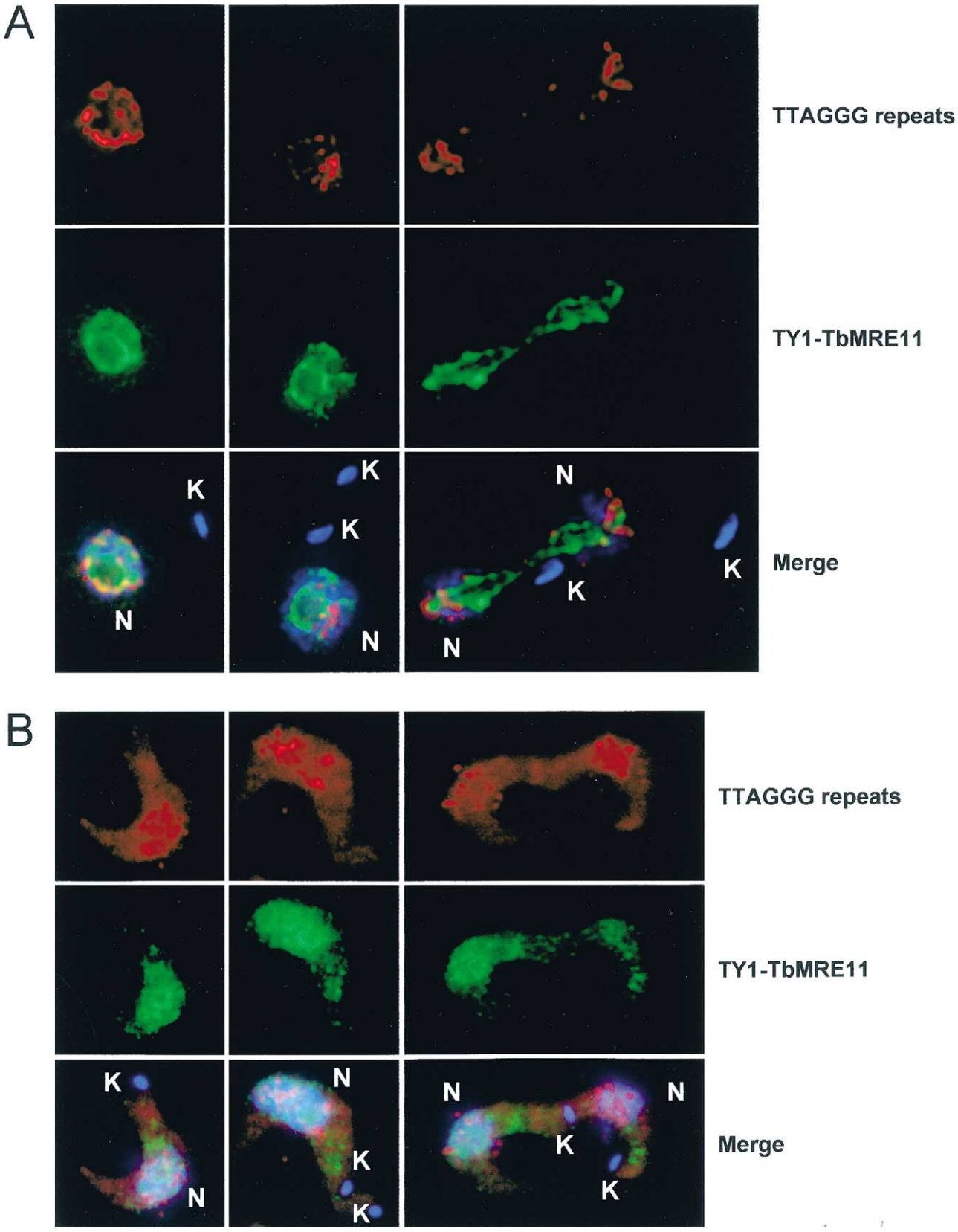
A
TTAGGG repeats
TY1-TbMRE11
Merge
K
N
B
TTAGGG repeats
TY1-TbMRE11
Merge

Fig. 2.

probe and observing under a conventional fluorescence microscope, we concluded that MRE11 did not co-localize to telomeres in bloodstream forms, but a number of procyclic forms showed partial telomeric co-localization. To ascertain if the apparent co-localization in procyclic forms was accurate, we employed deconvolution microscopy, obviating the risk of misalignment, which may occur when comparing different fluorescent probes. The deconvolution data confirmed that some *Tb*MRE11 was indeed located at or near telomeres. This association was most suggestive in cells that were in G1 phase (1K1N) [41] of the cell-cycle (Fig. 2A, left panel).

3.3. Generation and characterization of MRE11 KO mutants

The disruption strategy is illustrated in Fig. 3A. In wild-type *T. brucei*, *MRE11*$^{+/-}$ single-allele knockout mutants were easily obtained using the hygromycin cassette (100% of the clones assayed showed the expected integration). However, successful targeting of the second allele with a puromycin cassette was only achieved after several attempts, and then only one of 22 puromycin resistant clones possessed precisely the expected integration. The other 21 clones gave identical results on Southern analysis, indicating that the second *MRE11* allele had been targeted but incompletely deleted. A conditional null mutant expressing a tetracycline inducible *MRE11* was also established. This was achieved by introducing an ectopic Ty1-tagged *MRE11* into the *rDNA* locus of an *MRE11*$^{+/-}$ 'single/marker' cell line and transfecting the second knockout cassette during tetracycline induction, when the Ty1-MRE11 was seen as a distinct band at around 85 kDa on western blots (Fig. 3C). Upon removal of tetracycline from the conditional null mutant cells (*MRE11*$^{-/-/(+)}$), MRE11 was undetectable after 24 h, but low amounts were detected 80 and 116 days after tetracycline withdrawal (Fig. 3). This moderately leaky expression in the absence of tetracycline induction has been observed for similar pLew 82-based expression systems and does not seem to be specific to any particular gene. Despite this observation, the regulation of ectopic MRE11 was tight within the 1st week after tetracycline depletion, which allowed functional analyses within this timeframe.

3.4. Functional analysis of MRE11 in bloodstream-form T. brucei

Growth curves were generated for conditional null mutants in the presence or absence of tetracycline, and compared to Lister 427 wild type and single-allele *MRE11*$^{+/-}$ lines (Fig. 4A). The conditional null mutant grew more slowly, with an average population doubling time of 8 h, compared to parental cells that doubled every 6.5 h. The growth profiles of single-allele knockout and conditional null cells with tetracycline induction were difficult to resolve despite the cell culture being performed over extended periods (about 190 h). Subsequently, we employed colony growth on agarose plates to investigate if subtle growth differences can be visualized at shorter times. Fig. 4(B–D) shows that the conditional null mutant formed significantly fewer (55–65% of controls) and smaller colonies when compared to single-allele knockout and tetracycline-induced conditions. These effects were visible by day 5 post-inoculation. Plating also revealed that the growth defect in the mutants was probably caused by loss of viability among a fraction of cells in the population. In yeast and mammalian cells, MRE11 plays a pivotal role in DSB sensing and repair [42–44]. Repair may be facilitate by the homologous recombination pathway, and cells lacking MRE11 are sensitive to DNA damaging agents such as ionizing radiation or the radiomimetic alkylating agent MMS. We tested if the *MRE11* conditional mutants also displayed a phenotype consistent with the loss of active MRE11, by exposing the mutant cells to DNA damaging agents. Fig. 5(A,B) shows the viability of cells after exposure to MMS or ionizing radiation, respectively. At 0.00025% MMS, all the cell lines tested were mildly affected, with recovery in the range of 70–80%. However, at 0.0005% MMS, the conditional MRE11 mutant was significantly ($P \leq 0.02$) more susceptible (2% survival) than the parental line (30% survival), single-allele knockout (14% survival) and tetracycline-induced conditional null mutant (16% survival) lines. The ionizing radiation assay further corroborated the role of MRE11 in DNA damage control. At 500 rads, in the absence of tetracycline, the mutant showed a threefold reduction in survival; at 1000 rads the difference in survival rates was even more striking, with the conditional mutant showing approximately 10-fold reduction in the absence of tetracycline ($P < 0.02$). Here the addition of tetracycline fully restored the mutant to parental levels of resistance.

3.5. Homologous recombination

In *T. brucei*, integration of linear DNA into the genome occurs exclusively via homologous recombination [45]. We therefore investigated whether the absence of MRE11 would result in a deficiency in the integration of transfected DNA, targeted to the tubulin locus. MRE11 null mutant cells showed at least a 5-fold decrease in the ability to integrate a selectable DNA molecule into the genome (Table 1), after taking into account a 40% difference in the cloning efficiency of the null mutant compared to the wild-type control.

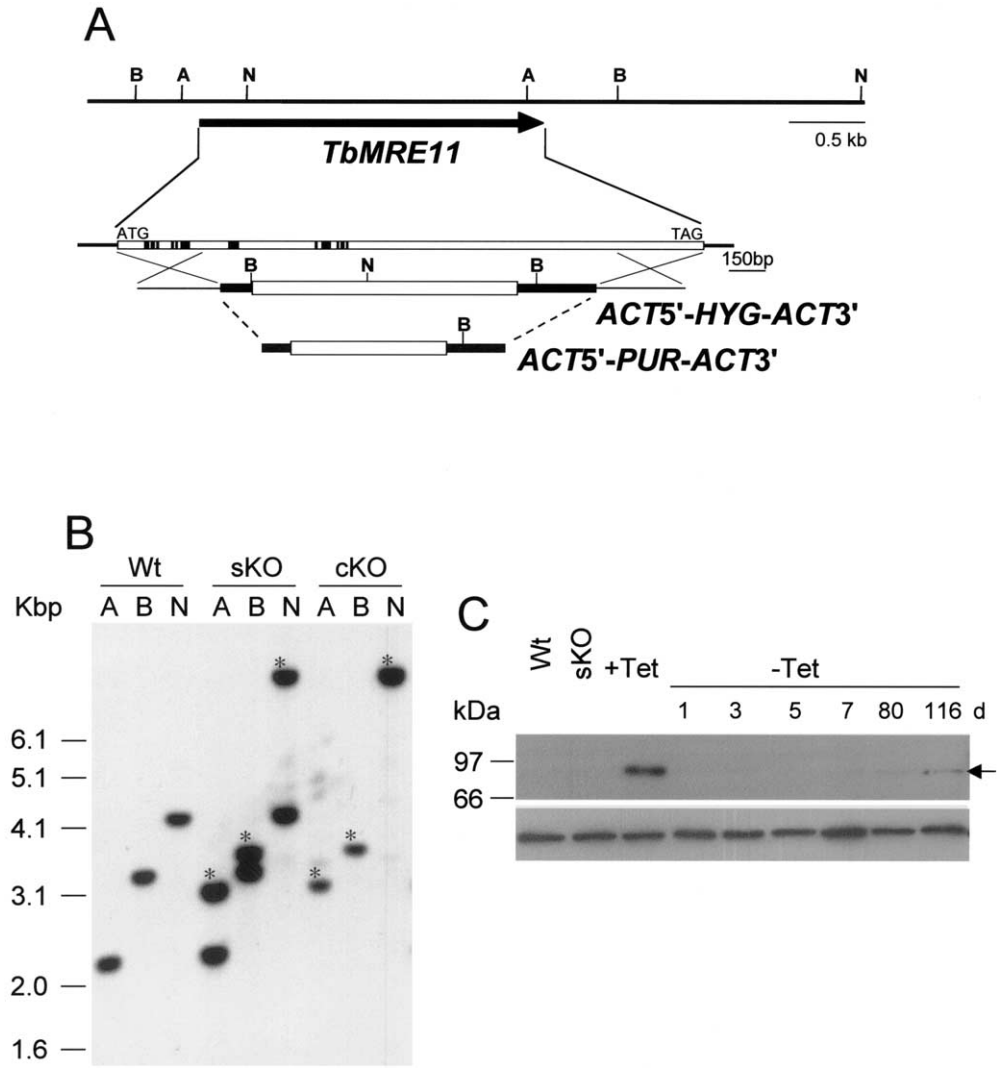

Fig. 3. Generation of *MRE11* conditional null mutant. (A) Genomic representation of the *MRE11* locus and disruption cassettes. Positions of highly conserved residues in the MRE11 amino acid sequence are shown as black vertical bars, and the 300-bp *MRE11* 5′ and 3′ region used for integration of the constructs are indicated with crosses. One thousand six hundred and twenty-six bp of the *MRE11* locus was replaced by the resistance cassettes that confer hygromycin or puromycin resistance and contain 5′ and 3′ sequences from the actin locus (gray shading). An ectopic construct, expressing a tetracycline-inducible T7 polymerase driven *Ty1-MRE11*, was inserted into a rDNA spacer region after single-allele knockout clones were obtained. A conditional null clone ($MRE11^{-/-(+)}$) was subsequently obtained in the presence of *Ty1-MRE11*. (B) Southern analysis of *MRE11*. Two micrograms of *T. brucei* genomic DNA was digested with diagnostic restriction enzymes (A, *Afl* II; B, *Bfa* I; N, *Nde* I). The probe contained *MRE11* internal coding sequence that only recognizes the endogenous and ectopic copies of the gene. Lister 427 wild type cell line (Wt) revealed a single band for each digest. Single-allele knockout cell line (sKO) expressing ectopic *Ty1-MRE11* shows bands corresponding to one endogenous allele and the ectopic gene (*). The conditional null cell line (cKO) only revealed bands corresponding to the ectopic gene. (C) Western blot using antibodies against Ty1 shows inducible expression of Ty1-MRE11 in the conditional null mutant line. Lane numbers represent culture period in days minus tetracycline. In the presence of tetracycline, a distinct band corresponding to the ectopic protein is arrowed. This band is barely perceptible at day 1 minus tetracycline but becomes more abundant after 80 days and increases only slightly at day 116. The lower band corresponds to a loading control using a monoclonal antibody (L8C4) against PFR A (a gift from K. Gull).

4. Discussion

This paper describes the identification and cloning of *T. brucei MRE11* and characterization of the encoded protein. The clear conservation of MRE11 sequences, predominantly in the N-terminal domain, strongly suggests that *Tb*MRE11 shares similar functions with yeast and mammalian MRE11. The N-terminal region of *S. cerevisiae* MRE11 contains four highly conserved phosphoesterase motifs, shown to be important for its nuclease activities. Point mutations within these motifs impair DSB processing, resulting in sensitivity to MMS [20] and ionizing radiation [40].

*Tb*MRE11 localizes, in a non-uniform fashion, to the nucleus of bloodstream and procyclic forms. From the immunofluorescence analyses, there appeared to be an accumulation of the ectopic protein in G2-M bloodstream forms. However, after extensive flow cytometry

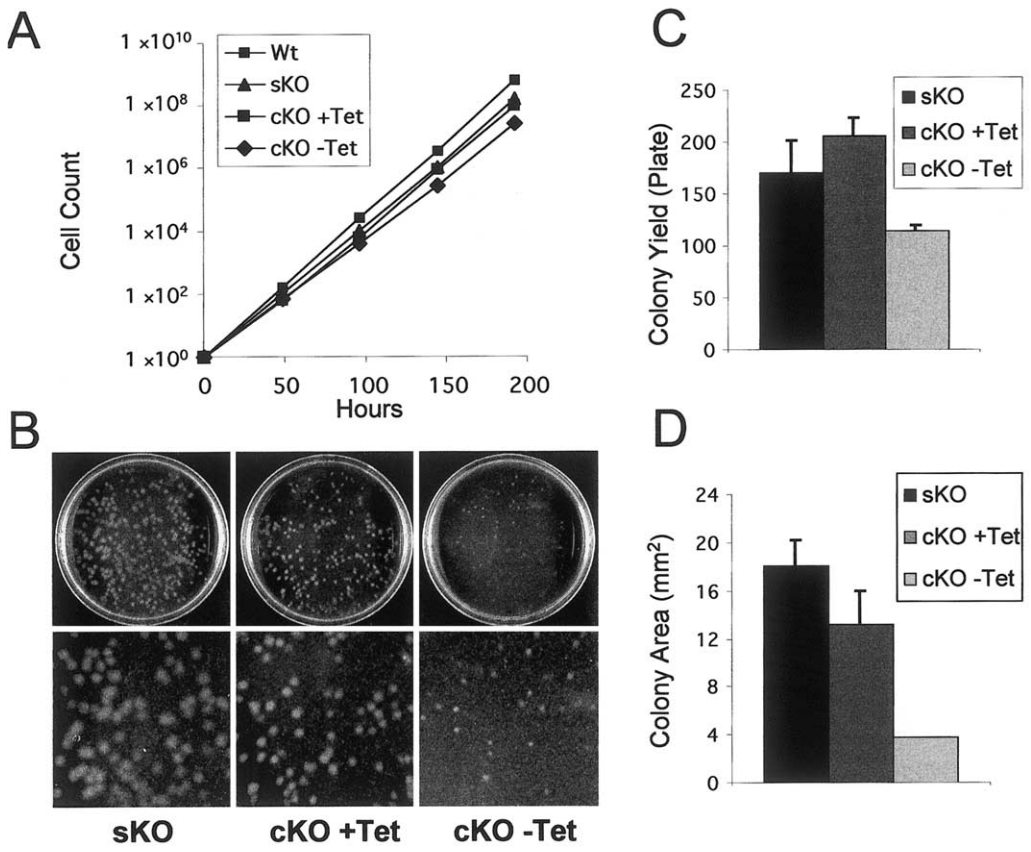

Fig. 4. Bloodstream-form *MRE11* conditional null mutant shows reduced growth in the absence of tetracycline. (A) Growth in liquid medium. At 48 h intervals, log-phase cells were counted then diluted to 1×10^4 cells ml^{-1}. The dilution factor for each cell line was used to calculate the cumulative cell yield of culture. (B) Colony growth of uninduced *MRE11* conditional null mutant cells compared to induced conditional null mutant and single-allele knockout cells and quantified by colony yield (C) and size (D). Error bars denote standard deviation from the mean of three separate experiments.

experiments (data not shown), using cells expressing other ectopic nuclear proteins (GFP-H2B and GFP-KU80) as controls, we concluded that the G2 increase was probably due to the doubling of DNA content and cell-cycle-specific synthesis or nuclear accumulation of MRE11, as the ratio of GFP-MRE11 to DNA remained constant throughout the cell-cycle.

However, the punctate sub-nuclear staining pattern seen in some G2-M cells may be significant. This may correspond to sites of DNA breaks or some other MRE11 requirement, as similar patterns have been observed for mammalian cells exposed to DNA damage [44,46], or at replication origins of S-phase cells [47]. Alternatively, the punctate staining may represent non-specific complex formation due to protein over-expression. This is unlikely because these discrete foci are readily apparent when MRE11 expression is induced with low (2.5 ng ml^{-1}) or high (500 ng ml^{-1}) tetracycline (not shown), suggesting that these foci are representative of wild-type cells. Interestingly, MRE11 shows partial co-localization with telomeres of procyclic forms. The use of deconvolution microscopy aided this assessment as images could be captured at high resolving powers and without the problem of misalignment that can be encountered with conventional fluorescence microscopy. The differences in the association of MRE11 with telomeres of procyclic and bloodstream forms might reflect differences in the chromatin of these two life-cycle stages. Bloodstream-form telomeres contain a substantial amount of a modified T, termed J which contain bulky glucose residues that may restrict the access of telomeric proteins [48]. The differences in MRE11 association may also reflect variations in telomeric chromatin structure, between the two life-cycle stages [49].

Down-regulation of ectopic *MRE11* resulted in a rather subtle growth phenotype in liquid medium. However, colony formation on agarose plates allowed us to distinguish various growth phenotypes and proved to be a time-saving technique in contrast to using liquid culture to generate growth curves. From the colony data, it is apparent that the growth phenotype may be predominantly or exclusively due to a reduction in cell viability. While this manuscript was in preparation, we became aware of another report [50] that also described growth retardation in *MRE11* null mutants of *T. brucei*.

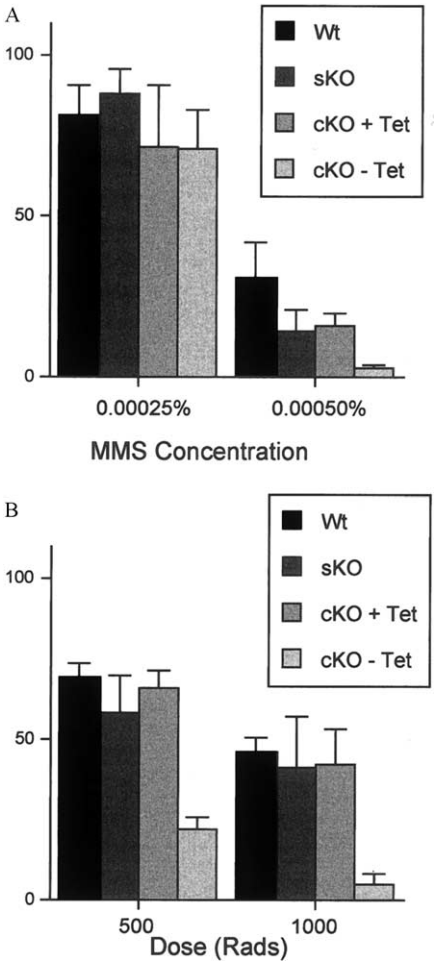

Fig. 5. *MRE11* conditional null mutant cells are more sensitive to DNA damage. (A) Cell lines exposed to 0.00025 and 0.0005% MMS. (B) The same cell lines exposed to 500 and 1000 rads of ionizing radiation. Bars represent viability of cells as a percentage of corresponding cells that were not exposed to the DNA damaging agent. Error bars denote standard deviation from the mean of four (MMS) and three (ionizing radiation) separate experiments.

Table 1
Homologous recombination assay

Strain	Number of clones
Wild type	86
$MRE11^{+/-}$	94
$MRE11^{-/-}$	9

Equal numbers of $MRE11^{+/+}$, $MRE11^{+/-}$ and $MRE11^{-/-}$ cells were transformed with pLew114, which targets a neomycin phosphotransferase gene to the β tubulin gene. Data represent the number of G418-resistant clones obtained from 11 transfections (per cell line) each with 1.5×10^7 cells. Results shown represent pooled values of three independent experiments.

This study also showed that *MRE11* null mutants undergo gross chromosomal rearrangements, and suggested that cell mortality was due to replication errors that had resulted in the loss of essential genes. Depletion of MRE11 is lethal in animal cells [51] and it has been suggested that MRE11 is crucial for the repair of spontaneous DNA breaks that arise during DNA replication [22]. However, components of the yeast MRE11 complex are not essential for survival, so much of the current information about MRE11 function has come from studies on *S. cerevisiae*. *Tb*MRE11 is clearly also non-essential, making *T. brucei* also an attractive system to study the functions of this molecule, particularly its potential role at telomeres. Trypanosomes have the same telomeric repeat sequence as vertebrate cells, and a single-stranded telomeric overhang of similar length to human cells. It was surprisingly difficult to disrupt the second allele, with only one out of 22 clones possessing precisely the expected integration event. The nature of the apparently similar insertion into the *MRE11* allele in the other 21 clones has not been investigated in detail, but it appears to be due to mis-targeting of one end of the construct, perhaps due to a short, unidentified region of additional sequence similarity. In budding yeast, MRE11 mutants are extremely sensitive to DSB inducing agents such as ionizing radiation and MMS. In this study, we similarly show that Tb*MRE11* conditional mutants are significantly more sensitive to ionizing radiation and MMS. Based on strong sequence homology and mutant phenotypes, it is likely that *Tb*MRE11 plays a role in the repair of DSBs. In contrast to our MMS sensitivity assays, the other recently published report on *Tb*MRE11 [50] showed that the null mutants were not significantly more sensitive to MMS, compared to controls. However, rather surprisingly, the mutants were hypersensitive to phleomycin, which induces DSBs. This puzzling finding may be due to differences between the maximum concentration of MMS used in the present (0.0005%) and the other (0.0004%) study. MRE11 also participates in the generation and processing of DSBs during meiotic recombination in yeast [20,40]. Genetic exchange has been shown to occur in *T. brucei* as it passes through *Glossina* [52]. This appears to be a rare event, and it remains to be seen whether there is a role for MRE11 during meiotic recombination in *T. brucei*. The highly conserved MRE11 N-terminus, containing regions essential for nuclease activity, has been shown to be involved in the exonucleolytic resection of 5′ ends to generate 3′ overhangs [20,40]. These overhangs participate in strand invasion of a corresponding homologous stretch of DNA on a sister chromatid that will be used as a repair template [53]. MRE11 could also play a role in generating the telomeric single-strand overhang. In *T. brucei* bloodstream forms, recombination events are involved in the most common route by which one expressed *VSG* is switched to another [25,54]. Of the different recombination mechanisms that have been described, the most common pathway involves a gene-conversion-like reac-

tion employing chromosomal-internal or silent telomeric VSG genes as donors [25]. It has been suggested that these gene conversion events are mediated by homologous recombination [25], evidenced by the observation that *T. brucei* mutants lacking the protein important for strand exchange, RAD51, are impaired in their ability to switch VSGs [29]. Surprisingly, MRE11 has very recently been reported not to play any role in *VSG* switching [50], although the rates of switching in this wild-type background were already very low, and the assay that was used would probably have resulted in a significant underestimate of switching rates.

The existence and high conservation of MRE11 in *T. brucei*, representing an early branched eukaryote, could provide valuable information on the primary functions of this interesting multi-functional protein. MRE11 plays a role in the DNA damage response in *T. brucei*, suggesting that at least one of its original purposes in the common ancestor was to repair DSBs. However, considering the bewildering number of functions MRE11 possesses in yeast and mammalian cells, further investigations should reveal other potentially interesting, and possibly novel, functions of MRE11 in *T. brucei*.

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