RESEARCH ARTICLE

The \textit{mre11} A470 alleles influence the hereditability and the segregation of telosomes in \textit{Saccharomyces cerevisiae}

In-Joon Baek, Daniel S. Moss, Arthur J. Lustig*

Department of Biochemistry and Molecular Biology, Tulane University Medical Center, New Orleans, Louisiana, United States of America

* alustig@tulane.edu

Abstract

Telomeres, the nucleoprotein complexes at the termini of linear chromosomes, are essential for the processes of end replication, end protection, and chromatin segregation. The Mre11 complex is involved in multiple cellular roles in DNA repair and structure in the regulation and function of telomere size homeostasis. In this study, we characterize yeast telomere chromatin structure, phenotypic heritability, and chromatin segregation in both wild-type \textit{[MRE11]} and A470 motif alleles. \textit{MRE11} strains confer a telomere size of 300 base pairs of G+T irregular simple sequence repeats. This DNA and a portion of subtelomeric DNA is embedded in a telosome: a MNase-resistant non-nucleosomal particle. Chromatin immunoprecipitation shows a three to four-fold lower occupancy of Mre11A470 T proteins than wild-type proteins in telosomes. Telosomes containing the Mre11A470T protein confer a greater resistance to MNase digestion than wild-type telosomes. The integration of a wild-type \textit{MRE11} allele into an ectopic locus in the genome of an \textit{mre11A470 T} mutant and the introduction of an \textit{mre11A470T} allele at an ectopic site in a wild-type strain lead to unexpectedly differing results. In each case, the replicated sister chromatids inherit telosomes containing only the protein encoded by the genomic \textit{mre11} locus, even in the presence of protein encoded by the opposing ectopic allele. We hypothesize that the telosome segregates by a conservative mechanism. These data support a mechanism for the linkage between sister chromatid replication and maintenance of either identical mutant or identical wild-type telosomes after replication of sister chromatosomes. These data suggest the presence of an active mechanism for chromatin segregation in yeast.

Introduction

The DNA-RNP structure present at the end of all eukaryotic chromosomes, the telomere, is necessary for end synthesis and protection. Our insight has grown into the polymerases, telomerase regulators, and chromatin components that solve the end-replication problem and contribute to the telomere end-protection [1]. The major mechanism to compensate for replicative attrition is the RNP-reverse transcriptase, telomerase [2]. Telomerase catalyzes the addition of G+T sequences onto pre-existing 3’ telomere ends. The activity of several stable
complexes, including telomerase, Ku70/Ku80 [3], CST [4–6], MRX [Mre11/Rad50/Xrs2][7, 8], Hoogsteen base-paired G4 DNA and G4 DNA binding proteins, protect the telomere [9–11]. A regulated competition between positive and negative factors creates a telomere homeostasis [12, 13] that in most eukaryotes acts as a dynamic telomere cap.

In all organisms, both Mre11 and Rad50 are dimeric, while NBS1 [or yXrs2] is monomeric. Together they form the MRX [or MRN] complex [14]. In the yeast model system Saccharomyces cerevisiae, the dynamic anti-checkpoint process begins with the binding of Tel1 [yATM] and Mre11 to only short telomeres [15–17]. Phosphorylation of telomerase by Tel1 results in the positive regulation of telomerase. In addition, Tel1 represses Rif1 [18], a protein that binds to the major telomere binding protein Rap1, and [19, 20] the single-strand DNA binding protein Cdc13 is activated by Cdk1[21, 22] phosphorylation. Both of these activities lead to a telomere size homeostasis through modulation of telomerase activity. These activation steps are in competition with Rif1 and Rif2, negative regulators of telomerase, that bind to Rap1 [5]. Rif1/Rap1 association increases the abundance of Rif1 in elongated telomeres. In contrast, Rif2 interferes with the activity of Tel1, leading to repression of telomere addition [23, 24].

Two types of subtelomeric elements are associated with the telomere tract, one or more 5.5 [short] or 6.7 [long] kb Y’ repeats, and the X-class telomeres that are also present telomere distal to the Y’ elements. The size of Y’ class telomeres can be easily determined by cleavage with Xhol, 870 base pairs [bp] from the telomere proximal Y’ terminus [25].

Telomeric chromatin structure [termed a telosome], unlike telomeric DNA, is partially protected against micrococcal nuclease [MNase] digestion. In wild-type cells the protected DNA fragment has a mean size of 400 bp [26]. In contrast, the telomeric tract is 300 bp in wild-type cells, suggesting that the telosome protects both subtelomeric and telomeric sequences. The duplex portion of DNA is slightly larger than the telomere tract and is bound, primarily, by Rap1 at specific sites once in every 18 bp of telomere tract [27]. The Rap1 crystal structure indicates that telomeric DNA threads through a "pore-like" structure, distinct from the nucleosomal wrapping of DNA [26, 28]. Additional proteins that bind to the telomere are also components of the telosome. In addition to the Rif1 and Rif2, Rap1 physically associates with Sir2/Sir4, and Sir3 heterochromatin [29–31]. We have used several characteristics of the telosome: chromatin structure, telomere size, and Mre11 association with telomeric chromatin to probe the linkage between heritability and segregation.

We have tested whether telomere chromatin [the telosome] segregates by random or conservative means. Conservative segregation is the maintenance of the same form of chromatin on both replicated sister chromatids [32, 33]. This results in the heritability of the phenotype associated with telomeric chromatin. We are particularly interested in the factors that are needed to maintain conservative segregation. The best-known case of conservative segregation is some classes of heterochromatin [34] that are continuously modified through rounds of DNA replication. Hence the accessibility of the chromatin to modification will not simply vanish after replication and can be “reseeded” during replication [35]. Reformation of histone modifications can explain, in part, conservative segregation. The mechanism, however, is still speculative and may differ in various types of heterochromatin, including telomeric regions. Similar modifications of both histones and telomere proteins are also found at human telomeres and may influence telomere length during development in human cells (e.g.[36, 37]). Non-histone modifications or histone/telomere proteins loops (e.g.[30, 38]) may also play a role in the yeast telosome segregation.

The structure of the first eukaryotic crystal of Mre11 [39] has facilitated research in the fields of telomere repair, recombination, and telomere size homeostasis. The highly conserved A470 motif consists of 13 consecutive amino acids [470–482] [40, 41] that, based on the crystal structure, are close to the Mre11/Rad50 interface [39].
In this study, we have used one allele in the A470 motif, mre11A470T, as a marker to separate heritability and telomere chromatin segregation [40]. Telosomes containing the Mre11 or Mre11A470T proteins display strikingly different MNase profiles. A cell population that produces only the mutant allele requires more time of MNase digestion than wild-type cells to cleave telomeres from chromatin. We show here that the association of Mre11A470T with the telosome is less than wild-type association. However, we cannot rule out an additional possibility that Mre11 or Mre11A470T also act in trans to influence telosome structure. We have compared telomere structure and hereditability phenotypes conferred by alleles located at the endogenous site [also called genomic site] with genes integrated at an ectopic site [Fig 1]. We have determined that only the product of the genomic locus is incorporated in the telosome and leads to heritability and conservative telosome segregation.

Materials and methods

Strains

All strains used in this study are isogenic to W303a [Mat a MRE11 leu2-3,112, ura3-1, trp1-1, HIS3, can1-100 rad5-353] or W303 α [Mat α MRE11 leu2-3,112 trp1-1 ura3-1 ade2-1 his3-11,15 can1-100 rad5-353] strains. KMM4 [Mat α mre11A470T trp1-1 leu2-3,112 ura3-1 his-11,153 can1-100 rad5-353] is a backcross between IJ-9c [MAT α mre11A470T trp1-1 ura3-1 1his3-11,15 rad-5-353] and W303a [40]. All strains were purified by sub-cloning prior to use. To construct the mre11 Δ null allele, we PCR-amplified DNA from an mre11: KanR cassette in the yeast strain BY4741 [Res. Gen. 95400.BY4741] and used this cassette to conduct a one–step replacement of the MRE11 gene with the mre11: KanR cassette in W303 strains. Metabolic and telomere size phenotypes verified the identity of the strain. The MRE11 gene integrates at a non-genomic [ectopic] locus and can complement a genomic null allele.

Fig 1. Telomere and telosome phenotypes in MRE11, mre11A470T, mre11Δ, and yKu70Δ strains. [A] Telomere size was determined by Southern blot analysis of XhoI-fractionated yeast DNA from MRE11, mre11A470T, yku70Δ, and mre11Δ cells using a telomere-specific probe. Wild-type strain had telomeres 300 bp in length, while both mre11 mutants had telomeres centered at 150 bps. The yku70Δ telomeres were centered at 200 bp in length. The samples were flanked by λ size markers [NEB]. [B] Telosome formation in MRE11, yku70Δ, and mre11A470T cells. Chromatin isolated from wild-type, yku70Δ, and mre11A470T cells was digested with one unit/100 μl MNase for the times indicated, fractionated, and subject to Southern blot analysis using the A750 probe. The control was performed under the same conditions in the absence of enzyme. The position of 500 bp using the λ size marker is shown on the left and right of the gel. A gel splice is present due to the elimination of any empty lane adjacent to the yku70Δ set of data. The percentage of the signal that represents telomeres is shown below the gel.

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The *rap1-5* allele of *RAP1* was generated as described [42]. Previous studies indicated that the integrated *rap1-5* allele has a weak temperature sensitive [ts] phenotype at 37˚C [43]. To test the genetic interaction between *MRE11* and *RAP1*, we constructed the double mutant, *rap1-5 mre11A470T*. All growth conditions with *rap1-5* strains were conducted at 25˚C, the permissive temperature, or 30˚C, the semi-permissive temperature, respectively.

**Media**

Strains in the absence of selection were grown in YPD media or YPD with adenine supplementation [YPAD], to a general facilitator of growth [44]. Strains containing the Kan^R^ cassette were grown in YPD + gentamycin. Strains auxotrophic for any metabolic marker was grown on synthetic minimal media containing the missing amino acid at standard concentrations [44].

**Rad5 function**

We tested the possible involvement of the *rad5-535* allele, present in the W303 genetic background, in the generation of *mre11A470T* phenotypes. Ubiquitin is a major regulator of repair occurring during transcription [transcription-coupled repair]. Our concern derives from our use of another major repair complex, Mre11/Rad50/Xrs2, that is responsible the response to double-strand DNA damage repair and to some cases of residual transcriptional-coupled repair [45]. Whether there is a relationship between ubiquitin in transcription coupled repair and/or double strand break repair remains unknown. We therefore took a conservative approach and tested the *MRE11* and *mre11A470T* strains for their dependence on the *RAD5* gene. To test the possibility that *rad5-535* influences the *mre11A470T* allele, the telomere size of a *rad5: Kan^R^ mre11A470T* double mutant was compared to single gene mutations. The short telomere length and heritability phenotypes of *mre11A470T* were not influenced by the *rad5-535* allele, indicating that the Rad5 is not involved in the telomeric Mre11A470T function. Wild-type strains containing only the *rad5-535* allele also had no influence on the telomere size phenotype and heritability [S1 Fig].

**Ectopic integration**

The EcoR1/Xba1 containing flanking DNA from *MRE11* and *mre11A470T* cells were cloned into pRS306 [46], creating pRS306-8 and pRS306-9, respectively. The *URA3* gene in pRS306 was cleaved by NcoI and transformed into strains carrying either *MRE11* or *mre11A470T* loci at the endogenous “genomic” locus [on chromosome XIII]. Integration resulted in Ura3^+^ at the site of recombination at the ectopic site [also called an “ectopic allele” [e]] on chromosome V. In contrast, all endogenous genomic loci were Ura3^-^ in the absence of the ectopic allele. All transformants contained a single copy of either *MRE11* or *mre11A470T*. Phenotypes were specified by the allele at the ectopic and genomic loci as e-*MRE11* g-*MRE11* [AJL551], and e-*mre11A470T* g-*MRE11* [AJL551], e-*mre11A470T* g-*mre11A470T* [AJL 553], and e-*MRE11* g-*MRE11* [AJL 554] strains.

**Ectopic assays for additional A470 motif alleles**

The additional A470 motif alleles used in this study, *mre11V471A*, *mre11E479A*, *mre11K480A* and *mreK482T*, were placed at the endogenous site by two step gene replacement of W303 with the pRS306-cloned alleles. These strains were integrated by homologous recombination of the MRE11 gene into the ectopic site [ura3] to form e-*MRE11* g-*mre11A471T*, e-*MRE11* g-
mre11E479A, e-MRE11 g-mre11K480A, and e-MRE11 g-mreK482T [designated AJL555-AJL558, respectively].

After integration of each A470 motif allele, we routinely retested for the presence of the mutation. To that end, we used the Mre11 forward primer, \[5' - CAAACGTATAGATAGATAGATA TACCCAAT - 3'\], and the Mre11 reverse primer, \[5' - GCTCCTCTCAAATGGCATACCTT G - 3'\] for PCR amplification and DNA sequencing.

**Measurement of growth rates and viability**

We measured the growth rates of independent MRE11, mre11A470T, rap1-5, and mre11A470T rap1-5 strains at three temperatures [23˚C, 30˚C, and 37˚C] in three trials. Cells were grown overnight at 30˚C and inoculated into 5 ml of YPAD media at the differing temperatures. The Cellometer Vision fluorescent counting system [Nexelcom Inc.] was used to visualize both viable and inviable cells. Propidium iodide is capable of entering and staining only inviable cells. Thus, viability is given as [(total number of cells – the number of propidium iodide-stained cells) / total cells] \times 100. Cell growth was measured in the absence of propidium iodide.

**Telosome analysis**

We analyzed telosome structure as described [26, 47]. Specifically, we aliquoted nuclei into 100 microliters [μl] MNase buffer with 1 mM CaCl₂. A predetermined amount of MNase, based on preliminary studies, was incubated with each strain tested at 30˚C as a function of time [0, 30, 60, 100, 140, 190, and 240 seconds [secs], and the reaction was terminated. Negative controls were treated identically, but lacked MNase. The chromatin fraction of each culture was isolated and analyzed by Southern blot analysis using the 750 bp fragment, purified from plasmid Ap135 [A750, kind gift of Dr. Alison Bartuch], as the telomere probe. One of three size markers [the lambda size ladder, 1 [kilo-base] kb ladder and the 100 bp ladder [NEB] flanked each of the gels. Since chromatin experiments display subtle variations among independent experiments, we interpret the results of each experiment with all samples conducted in parallel on a quantitative basis, rather than the mean of data generated in differing experiments. Nonetheless, the pattern of size distributions is qualitatively reproducible between experiments.

**Telosome quantification**

Each lane of one set of gels was scanned by the CLIQ software [Total Lab] and the fragments quantified using the “rubber band” background setting per manufacturer’s instructions. High and low signals [in pixels] should be proportional to their abundance, since tract sizes are not the major variable in determining the increases in hybridization intensity [unlike measurements of telomere tract size]. The total pixels remain relatively constant [+/- 15%]. The intensity of telomere pixels/ the sum of all fragments gave rise to the % of the signal within the telomere size range. In some cases, the distribution varied slightly, and a broader range of a specific fragment was converted after increased treatment to a single species.

**Telomeric DNA isolated from telosomes**

We isolated DNA from the chromatin fraction by phenol: chloroform extraction and ethanol precipitation. Samples were digested with Xhol to determine telomere size after Southern blot analysis using the A750 as a probe. We determined the telomere size using the Y’ end-point at 870 bp from the Xhol site to terminus of Y’ [48].
Cycloheximide [CHX] protein stability assays

The CHX protein stability assay was performed in MRE11 and mre11A470T strains. In the first day, the cells were pre-inoculated overnight in YPD and re-inoculated in YPD to newly prepared YPD media after overnight growth. The cells were grown to mid-log phase, and then 100 μg of CHX was added, incubated with CHX for 0, 15, 30, 60, 90, 120, 160, 150, 180, and 240 minutes [min] at 30˚C. The proteins were extracted by the TCA method [49]. For Mre11 protein detection, a diluted [1/1000] primary Mre11 antibody [Rabbit polyclonal to Mre11-ChIP Grade, ABCAM ab:12159] was bound for one hour at RT and membrane was washed with TTBS buffer [a mixture of Tris-buffered saline [TBS] and Polysorbate 20 [Tween 20]] three times for 5 min at RT. The 1/2000 diluted secondary antibody [anti-rabbit, Santa Cruz sc:2313] was bound for one hour at RT and washed with TTBS buffer four times for 5 min at RT. The Mre11 proteins were detected by Western Blotting Luminal Reagent [Santa Cruz; sc:2048].

Reverse transcriptase Real-Time PCR for Mre11 transcript abundance

RNA was extracted from g-MRE11, g-MRE11e-MRE11, and e-mre11A470T g-MRE11 strains using phenol: chloroform: isomyl alcohol and subsequent ethanol precipitation. The cDNA was synthesized from one microgram of RNA after addition of four μl of I-Script RT Supermix [Bio-Rad], and the mix was diluted to 20 μl with dH2O. After primer extension, the reaction was incubated in the Bio-Rad complete mix for five min at 25˚C, and with reverse transcriptase for 30 min at 42˚C. Reverse transcriptase was inactivated for one minute at 95˚C. The qPCR, iTaq™ Universal SYBR® Green Supermix [Bio-Rad] was used for CFX96 Touch™ Real-Time PCR Detection System [Bio-Rad]. The primers used are, MRE11 F: ATGGTTGCGGAATTACC G, MRE11R: CCAACTTCTGGTAATAAAGAT AG, ACT1 F: GTCCCAATTGCTCGAGAG ATTTCT, and ACT1 R: GACCATGATACCTTGGTGT CTTGG.

Chromatin immunoprecipitation [ChIP] methodology

We followed the procedure of Hecht et al [31] using the following modifications: Cultures were scaled to 20 ml, and cells were incubated with 1% formaldehyde for 15 min at 37˚C before quenching. Cells were washed twice with cold PBS and suspended in collection of the lysate. Cells were sonicated three times for four secs at a setting of five [Dismembrator Model 100, Fisher]. The pellets were then centrifuged, and the supernatant containing the extract was collected. The immunoprecipitation used the ChIP-grade anti-Mre11 antibody [1/150] incubated overnight at 4˚C on a rotator. We pre-equilibrated the protein [A/G] agarose beads [Santa-Cruz; sc:2003] for two hours [hrs] at 4˚C. We subsequently washed the beads twice with 500μl lysis buffer at room temperature*. The fragments were eluted from beads with 50 μl 100 mM NaHCO3, 1% SDS at 65˚C for 15 min. Centrifugation separated the beads from the eluate. 50 μl TE and 100 micro-grams [μg] proteinase K were added, and precipitated by ethanol. The final DNA was dissolved in 30 μl TE and subjected to PCR using primers flanking a 200 bp sequence to amplify the X/telomeric border using the Xforward primer 5’−GGAGCAACTTG CGTGAATCGAAGA−3’, and the Telreverser primer: CGTTCGATGATGCTGCTAAA CTG. Lysis buffer = [HEPES/KOH [50 milli-molar [mM] pH7.5], 500 mM NaCl, 1 mM EDTA, 1% Triton X-100, DOC [0.1%], SDS [0.1%] and a protease inhibitor cocktail [Santa Cruz; SC29131] dissolved in 30 μl TE.

Real-Time PCR ChIP analysis of Mre11 abundance in telomeric chromatin

Cells were grown to OD600 = 0.2 in 20 ml of fresh liquid media to mid-log phase. Real-time qPCR was conducted according to the manufacturer’s instructions [Bio-Rad] as summarized.
below. We cross-linked proteins in vivo using a 1% formaldehyde for 15 min in a shaker at room temperature. The formaldehyde reaction was then quenched with 125 mM glycine. The cells were washed twice with cold PBS and suspended in 400ul lysis buffer. Cells were lysed with glass beads in a bead-beater four times for 20 secs each, transferred to a new tube and sonicated twice for 10 secs. The lysate was collected in a new tube and centrifuged for 10 min at 4˚C. We immunoprecipitated the supernatant overnight at 4˚C with a 1/150 antibody dilution on a rotator. We subsequently added 25μl of a 50% slurry of the pre-equilibrated protein A/G. Agarose beads were incubated for two hrs on a rotator at 4˚C. The beads were pelleted and washed with twice with 500 μl lysis buffer for two times at room temperature, once with 500 μl deoxycholate buffer beads [10 mM Tris ph7.5 0.25 M LiCl and 0.5% sodium deoxycholate and 1 mM EDTA] at room temperature, and once with 500 μl, TE for five min. The fragments were eluted from beads at 65˚C in 50 μl elution buffer for 15 min. The supernatant was collected and transferred to a new tube. The cross-links were reversed after incubating at 65˚C in 50 μl elution buffer overnight.

The beads were removed from the supernatant, and 50 μl TE and 100 μg proteinase K were added and incubated for three hrs at 55˚C. One hundred μl TE and 200 μl PCIA were added, mixed well and centrifuged for five min at room temperature. The supernatant was ethanol precipitated for 30 min at -20˚C, and the precipitate isolated after spinning at 14,000 x g for 30 min at 4˚C. The pellet was dried and dissolved in 300 μl TE. Procedures are accessible to the public by the MIQE convention [50].

We conducted real-time qPCR [Bio-Rad] using the CFX96 Touch™ apparatus and the primers Xforward and Telreverse described above. The data were analyzed using the Bio-Rad CFX Manager™ Software and the ΔΔC value was derived. Cq values from the CFX Manager software were used to determine the ΔCq [Target Cq- reference Cq], ΔCq expression [2^-ΔΔC], mean ΔCq expression [average replicates], ΔCq expression standard deviation, and the ΔΔCq [normalized value to mre11Δ]. The ChIP analysis was carried out as before, and reverse crosslinking performed in a final volume of two microliters of ChIP DNA using two different dilutions of protein in 96 well plates that were subjected to qPCR.

Telomere sequencing
We sequenced telomeres by the C-tailing method using the 5’ GTTCTCAGAAATTCT TCATT CGTAG-3’ [C-tail primer] and the unique telomere-proximal X-class primer 5`-GAGTTGG ATACGGG TAGTTGG-3’ that shares homology with 13 termini [51]. Sequencing was carried out by Fasteris, Inc. [https://www.fasteris.com].

Results
Heritability and telosome structure in mre11A470T cells

The altered telosomes of mre11A470T alleles. MRE11 and mre11A470T telomere tract lengths were measured by gel electrophoresis of XhoI sensitive cleavage Y’ elements that are 870 bp proximal to the telomere. The lengths were 300 bp and 150 bp, respectively, in Y’ and X [40] classes and packaged in telomere chromatin particles, termed telosomes [Fig 1]. After MNase treatment of chromatin, wild-type cells protect a telosome DNA fragment of 400–600 bp, as reported [26]. In contrast, the mre11A470T allele telosome is more resistant to MNase and protects a more diffuse pattern of subtelomeric sequences [100–900 bp]. Quantifying the signals leads to the conclusion that the mre11A470T telosome is present at maximal abundance after 240 secs of digestion, rather than 140 secs for wild-type telomeres. These data indicate that the telosome extends into subtelomeric sequences.
Micrococcal nuclease resistance phenotype. The mre11A470T allele is more resistant than wild type to MNase. In the mre11A470T allele, extended times of digestion are required to reach the point of telosome release from chromatin. These data imply that the structure or folding of Mre11A470T and associated proteins protect telosomes from digestion. The delay in cleavage of mre11A470T chromatin is not a consequence of short telomeres, since yku70Δ telosomes has a wild-type level of resistance [Fig 1B]. In contrast, the mre11 null allele confers a variety of chromatin structures from wild type to mutant, and may reflect multiple states of the telosome in the absence Mre11. As expected, DNA isolated from the chromatin fraction gave rise to the initial telomere sizes [S2 Fig].

The effect of altered telosomes on telomere sequence. A change in the relative position of the telomeres may be the consequence of Mre11A470T-induced changes in the TLC1 telomerase RNA template region. The telomere sequence could be altered or constrained in the mutant strain. We therefore sequenced multiple independent clones of both wild type and mre11A470T strains. However, we find no changes in sequence or sequence structure. The sequences of both the wild-type, [S3 Fig] and mre11A470T DNA [S4 Fig], vary between subclones, but follow the yeast consensus sequence G1-3T. Wild-type telomeres were conserved in the first 150 bp sequences distal to the terminus, but varied significantly in the terminal half due to deletions and sequence mismatches.

ChIP analysis of MRE11 and mre11A470T strains. To determine whether the Mre11 and Mre11A470T proteins are capable of binding to the telosome, we conducted a simple chromatin immunoprecipitation [ChIP] analysis using excess Mre11 and Mre11A470T [41] [Fig 2A, top]. Mre11 proteins were present as intact species of known size [80 kD] after Western analysis using the monoclonal antibodies directed against a C- terminal peptide of Mre11 [Abcam].
We crosslinked proteins in strains containing \textit{MRE11} or \textit{mre11A470T} alleles, and we immunoprecipitated cellular extracts with the same anti-Mre11 antibody. After elution, de-crosslinking, and protease treatment, a DNA was released that could support the PCR amplification of a 200 bp product at the junction between the X element and telomeric sequences. This fragment is maintained within the telosome, based on telomere size analysis. The \textit{mre11A} allele, on the other hand, generated no protein species or amplified DNA after ChIP analysis [Fig 2B]. These binding data reinforce that both Mre11 and Mre11A470T proteins are a part of the telosome.

To estimate the relative binding of Mre11 and Mre11A470T to the telosome at X class telomeres, we performed real-time qPCR ChIPs [Fig 2C]. DNA isolated from chromatin that was immunoprecipitated by anti-Mre11 [ABCAM] in \textit{MRE11}, \textit{mre11A470T}, or \textit{mre11A} extracts. The immunoprecipitated DNA was subject to real-time PCR to quantify the 200 bp junctional fragment [as a proxy for telomeres] using the same PCR primers as described above. We found a 3-4-fold increased abundance of telosomes containing wild-type Mre11 over Mre11A470T from wild-type and \textit{mre11A470T} cells.

An alternative explanation for the ChIP data is a four-fold decrease in the stability of the mutant protein. To test this possibility, we conducted a cycloheximide [CHX] translational block, and examined the subsequent time points for protein degradation. Both wild-type and mutant Mre11 proteins had identical high stabilities, similar to the values of the actin loading control [S5A Fig]. We can conclude therefore that both proteins are bound and stable at the telomere chromatin.

Phenotype and structural characteristics of alleles at ectopic and genomic positions

Epistatic relationship between \textit{MRE11} and \textit{mre11A470T} alleles in heritability and chromatin structure. In considering the ectopic/genomic system, we first had to resolve whether the allele at an ectopic site is silenced. Several lines of evidence argue against this possibility. First, a TAP-tag derivative of both wild type and \textit{mre11A470T} was integrated at an ectopic site and produced proteins that are also stable in a CHX assay [S5B Fig]. Second, the allele at the ectopic site can be expressed, since the wild-type allele at the \textit{URA3} locus on chromosome IX can complement an \textit{mre11}: \textit{Kan} \textsuperscript{R} allele at the genomic site [see 4, right]. Third, based on real-time qPCR analysis, reverse transcription of genes located at both genomic and ectopic sites have a three- to four-fold increase over either strain carrying the genomic allele alone [S6 Fig]. These data suggest that the genes at ectopic and genomic sites are both transcriptionally active. Hence, the ectopic sites produce transcripts and intact tagged proteins.

Strains carrying the wild-type gene at the endogenous genomic locus [g] were transformed and integrated with the \textit{mre11A470T} gene at an ectopic [e] \textit{ura3} locus. Conversely, strains carrying the \textit{mre11A470T} allele at the genomic locus were integrated with \textit{MRE11} at the ectopic \textit{ura3}. The only phenotypes observed were the result of the gene location at the endogenous locus [Fig 3].

Both the telomere tract length and MNase digestion pattern of chromatin were analyzed in e-MRE11 g-MRE11 and e-\textit{mre11A470T} g-MRE11 strains. In the opposing experiment, strains were analyzed in e-\textit{mre11A470T} g-MRE11 and e-\textit{mre11A470T} g-\textit{mre11A470T}. We will term this type of strain as “ectopic/genomic” to reflect the positions of the two alleles. The heritability of short mutant telomeres in e-MRE11 g-\textit{mre11A470T} and g-\textit{mre11A470T} strains persisted over 100 generations [the longest period tested]. In contrast, the wild-type telomere lengths persisted in e- \textit{mre11A470T} g-MRE11 and g-MRE11 strains [Fig 4].

We found similar MNase patterns and spacing [diffusion of chromatin] isolated from e-MRE11 g-\textit{mre11A470T}, and e-\textit{mre11A470T} g-\textit{mre11A470T}, and isolated from e-\textit{mre11A470T}
g-MRE11 and e-MRE11 g-MRE11 cells sub-cultured for the same number of generations [Fig 5]. This is similar to single mutants mre11A470T [maximal extent: 100 bp-900 bp] and yku70Δ [maximal extent 100 bp-900 bp] telosomes [Fig 1B]. In one case, an mre11A470T gene that integrated into an ectopic site in a MRE11 strain [Fig 5, [e-mre11A470T gMRE11; 240 min]] produced a “diffuse” wild-type telosome [maximal extent: 200 bp-1000 bp]. Although insufficient to make a statistically sound conclusion, the diffusion phenotype is biased towards short telomeres. These diffuse telomeres are likely to represent a broader range of MNase cleavage sites that are less defined to a specified size range. Thus, in most aspects, the final phenotype is close to the genomic locus, regardless of the presence of opposing alleles at ectopic sites. As a control, e-MRE11 complemented a null allele.

Assays of additional A470 alleles reveal their dominant epistasis when the A470T allele is at the genomic site and the MRE11 allele is at the ectopic site. Each A470 motif allele confers either shorter telomeres [V471A, E479A], that [similar to the mre11A470T allele,] [Fig 6] or larger intermediate telomere sizes [K480A, A482T] that have equivalent telomere sizes in the presence or absence of the wild-type allele. These data demonstrate that a common trait of A470 alleles confine the phenotypic expression to the genomic loci.

Rap1 and Mre11 interaction

Rap1 and Mre11 mutant proteins confer synthetically lethal at 37˚C. We have shown that Mre11 can be defined both as part MNase nuclease sensitivity and by ChIP analysis. The telosome has previously been characterized as a component of the telomeric chromatin [26, 47, 52]. Although we do not know the relative degree of association of each component, we will assume they are generally part of the same telosome particle. We therefore tested the genetic or physical association of Rap1 and Mre11 at a basic level. To this end, we examined the growth and viability characteristics of a double mutant containing the mre11A470T and a weak temperature sensitive [ts] allele of RAP1, rap1-5. The rap1-5 was chosen, in place of more severe rap1 alleles, since rap1-5 defects would allow changes in double mutants to be observed before lethality. MRE11, RAP1, MRE11 rap1-5, mre11A470T RAP1, and mre11A470T rap1-5 cells were assayed for both cell growth viability and cell growth rate. Viability [as measured by
the % of viable cells] was tested for cells at 23˚C, 30˚C and 37˚C, reflecting the permissive, semi-permissive and restrictive temperatures of \textit{rap1-5}. Viability was not altered at 23˚C and 30˚C in any strain, while \textit{mre11A470T} grown at 37˚C decreased viability to 90%, and the double mutant to 60% [Fig 7]. [The \textit{rap1-5} gene did not confer a viability phenotype over this temperature.

Fig 4. Telomere size and heritability in ectopic/genomic strains. [A] Telomere size homeostasis in e-M\textit{RE11} g-m\textit{re11A470T}, e-m\textit{re11A470T} g-M\textit{RE11}, e-M\textit{RE11} g-M\textit{RE11} and e-m\textit{re11A470T} g-m\textit{re11A470T} strains before [left] and after 100 PDs [right] [10 cycles of continuous subculturing with 10 population doublings each] of growth. M\textit{RE11} [lane 2] and m\textit{re11A470T} [lane 11] were also used as controls before and after sub-culturing. Markers flank the samples. Arrows designate the wild-type and mutant length of the telomeres. The identity of samples that were derived from DNA before and after sub-culturing are designated beneath the gel. [B] To check whether the wild-type gene at an ectopic site still behaves phenotypically as a wild type, despite an \textit{mre11Δ} null allele, genomic DNA was extracted by phenol: chloroform: isooamyl alcohol extraction, precipitated with ethanol, digested with \textit{XhoI} and subjected to Southern Analysis using the A750 probe. The e-M\textit{RE11} allele complements the short telomere phenotype of a \textit{mre11Δ} strain, indicating that the ectopic site is expressed.

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Fig 5. Chromatin characterization from ectopic/genomic strains. Telosome formation of e-M\textit{RE11} g-M\textit{RE11}, e-m\textit{re11A470T}, g-m\textit{re11A470T}, e-M\textit{RE11} g-M\textit{RE11} and e-m\textit{re11A470T} g-m\textit{re11A470T} were prepared as described in 2. The chromatin was extracted and digested by one unit/μl MNase at the designated time points. The gel was subjected to Southern blot analysis using A750 as the probe. The percentage of the signal that represents telomeres is shown below the gel lanes.

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short time interval]. This non-epistatic relationship between the viability conferred by the
typeos of mre11A470T and rap1-5 suggests that Rap1 and Mre11 act independently on the
telomere through different pathways or form a toxic complex that decreases viability.

Rap1 and Mre11 pathways influence viability [Fig 7] and growth rate [Fig 8] in distinct fashions. Growth rates were more significantly affected. At all temperatures, mre11A470T rap1-5 and mre11A470T cell growth rates were reduced, with the double mutant having the most severe phenotype at each temperature [Fig 8]. The rap1-5 strain showed no change in viability at any temperature within this time frame [Fig 7]. Only the mre11A470T and mre11A470T rap1-5 strains were decreased in viability, albeit after differing times. Yet, cells stop growth before viability is lost, suggesting a window in which other events after growth
cessation can still influence viability. Given the proteins involved, it is likely that either the lack
of viability or the length of the cell cycle is the consequence of telomere-mediated genomic
instability.

Chromatin structure of rap1-5 mre1A470T telomeres. To correlate growth rate with the
telosome structure of the double mutant, we conducted a Southern blot of the products of
MNase digestion in wild-type, mre11A470T, rap1-5 and mre11A470T rap1-5 cells grown at the
semi-permissive temperature, 30˚C [Fig 9]. Both the mre11A470T rap1-5 and mre11A470T
chromatin are relatively resistant to MNase until 100–240 secs, when both rapidly form telo-
somes. Wild-type and rap1-5 cells start to form telosomes at an earlier time than mre11A470T.
Hence, rap1-5 does not facilitate more resistance to MNase. This is consistent with the small
decrease in growth rate at 30˚C in mre11A470T cells and the double mutants. We are some-
what disadvantaged by the inability to analyze chromatin structure at 37˚C, given the high lev-
els of unviability. This difference in sensitivity of different samples to reaction conditions of a
single experiment is less than the difference in sensitivity to inherent variability between exper-
iments. Nonetheless, the same qualitative pattern is maintained between identical experimen-
tal trials.
Discussion

Several lines of evidence indicate that telosome structure, hereditability and segregation are aberrant in the presence of the mre11A470T allele. First, an extended time of MNase cleavage is required to generate the Mre11A470T telosome. Real-time qPCR has shown a four-fold greater abundance of Mre11 than Mre11A470T in unsynchronized cells. This difference is not due to variations in protein stability.

Second, the mutant may not just alter structure, but could also facilitate the influence of a chaperone or regulatory RNA, such as hsp90 [53] and TERRA [54, 55], respectively. TERRA in yeast is currently an experimental ‘black box’ that may be the key to understand higher-level G-4DNA/telomeric DNA complexes [56].

Third, we have previously shown that the mre11A470T allele favors recombination over telomerase activity at chromosomal telomeres, based on the inability to heal a telomere seed sequence [41]. Indeed, ctf18 mre11A470T cells can confer a complete loss in telomerase activity in vivo [41]. This finding is consistent with the bypass of telomerase senescence in mre11A470T tcl1A, the apparent consequence of elevated telomere recombination [35]. One speculative possibility is that mre11A470T requires a cofactor to act directly on recombination. Alternatively, the structures of 3’ termini may be a deciding factor in the activation of telomerase through the binding of different factors that influence heritability, telosome segregation, and genomic stability.

Finally, since Mre11 is dimeric, the conservative segregation pattern of telosome observed in mre11A470T cells must be related to the Mre11 and Mre11A470T homodimer and heterodimer stability. Alternatively, the absence of a chromatin modification may prevent the...
incorporation of heterodimers. This possibility has precedent from positive and negative effects on transcription elicited by histone modification within a microenvironment (e.g., [36, 37]).

The heterodimer paradox

The lack of a phenotype generated from the ectopic locus is paradoxical since both genomic and ectopic alleles are expressed. MRE11 at an ectopic site can complement genomic mre11Δ alleles, the combination of the genomic and ectopic loci increases the steady state level of transcription, and a Tap-tagged MRE11 or mre11A470T can be expressed at the ectopic site. Proteins encoded by MRE11 or mre11A470T at the genomic and ectopic sites are also equally capable of translation [S5 Fig]. For example, heterodimers must be different, depending upon the genomic allele, in order to produce only two possible phenotypes from all allelic
combinations. Specifically, the e-mre11A470T g-MRE11 (1) and e-MRE11 g-mre11A470T (2) should both produce a mre11A470T/MRE11 heterodimer. If so, they have distinct phenotypes. Possibly, Mre11/Rad50 interaction, 3' end formation, or heterodimer instability interfere with the production of heterodimers.

Conservative segregation, the heterodimer problem and heritability

A conservative segregation pattern is a general requirement for telomeric chromatin, regardless of the specific mechanisms involved. There is precedence for conservative segregation or spreading of heterochromatin that is caused by specific base modifications [57].

In order to explain our results, we must assume that heterodimers are dysfunctional in an activity or in stability. Alternatively, there may be a strong selection for homodimer formation on telosomes. Telomeres have non-redundant mechanism of telomere cohesion, and may be specific to sister chromatid cohesion. Thus, separation occurs first at G2. Possibly, alignment of sister chromatids contributes for cohesin-mediated transient sister chromatid separation in G2 [Fig 10]. Alternatively, a closer proximity of the sister alleles may help to maintain the pre-existing state.

Characterization of Mre11A470T

The mre11A470T allele confers a unique chromatin structure. The telosome that contains the Mre11A470T protein requires longer MNase incubation for the release of the mutant particle from the total chromatin. Also, relative to wild type, the distribution is more diffuse than expected. This distribution suggests conformational changes that result in promiscuous subtelomeric MNase cut sites. However, this argument oversimplifies the mechanism since we have identified an expected wild type phenotype that is diffuse. The source of this property remains unknown. However, one speculative mechanism is a region of lowered stability or altered conformation, such as a fold-back structure [38], formed between an mre11A470T telomere and the subtelomeric region. The fold-back structure may have a lowered fidelity of MNase cut sites, and as a consequence, a broad range of telomere sizes after digestion.
Rad50/Mre11 interactions

One of the reasons for our interest in the Mre11A470T protein is its proximity to Mre11/Rad50 interface in eukaryotic crystal structures [39]. Indeed, the “dominant” epistatic pattern is a common feature of the A470 alleles. In each case, a wild-type gene integrated at an ectopic site failed to produce wild-type telomeres. This feature suggests their common involvement of the A470 motif in related functions, possibility regulating the Mre11A470T/Rad50 structure.

Synergistic effects of rap1-5 and mre11A470T

The rap1-5 mre11A470T double mutant is synergistic at all temperatures in growth rate, and at 37˚C in viability. Rap1 and Mre11 appear to act on the same substrate through differing pathways, both of which affect growth rate. Pathways are less clear in the telosome profiles at 30˚C, in which mre11A470T is epistatic to rap1-5. Mre11 may play a role in the equilibrium between Tel1 and Rif1 and in size homeostasis [16, 59]. Mre11 may participate in the formation of homeostasis, while Rap1 may play a role in protection against non-homologous end-joining [60, 61]. Experiments that examine the dynamics of wild-type strains are needed to resolve this issue.

Supporting information

S1 Fig. To investigate effect of RAD5 on telomere size, RAD5 was deleted in MRE11 and mre11A470T strains. XhoI digestion of DNA from the following genotypes were tested: Lane1, MRE11 RAD5; lane2, mre11A470T RAD5; lane3, MRE11rad5Δ; and lane 4 is mre11A470T rad5Δ strains. The rad5Δ deletion did not give rise to a telomere size change. The mre11A470T rad5Δ double mutant has the same short telomere phenotype and heritability as mre11A470T cells. (TIF)

S2 Fig. Telosome derived telomere size. We extracted DNA from WT, mre11A470T, mre11Δ, mre11A470T rap1-5, and mre11Δ rap1-5 chromatin. The DNA was digested with XhoI and
subjected to Southern blot analysis using the A750 probe. Two exposures are shown to visualize all fragments.
(TIF)

**S3 Fig. Telomere sequencing in mre11A470T strains.** We sequenced multiple X-class telomeres from independent clones of wild-type or mre11A470T cells using the C-tail method [51] with the X-class primers adjacent to a telomere tract. The X-class telomeres represent a group of 13 telomeres that have identical junctions with the telomere. S3A: Sequence of three clones of a mre11A470T was conducted on independent telomeres derived from a single source. The color code is present simply for convenience and is not meant to indicate causality.
(TIF)

**S4 Fig. Multiple clones shown at telomere-proximal and distal sequences of a wild-type strain.** The top sequences run from the telomere proximal to distal direction. The color code denotes the type of misalignment or gap among different telomeres even when they were in the minority. The comparisons are not used to determine causal relationships, simply the type of events that are occurring.
(TIF)

**S5 Fig. Cycloheximide stability assays.** [A] Stability assay for Mre11-Tap and Mre11A470T-A portion of the CHX stability assay of strains containing a locus at the genomic site and TAP-tagged derivatives [Mre11-TAP and Mre11A470PT-TAP]. Westerns were probed with anti-TAP antibody antibodies as described in part B. The protein migrates more slowly due to the TAP tag. Size markers are provided on the left. The blot was stripped and re-probed with anti-actin antibody. [B] CHX protein stability assay was performed in MRE11 and mre11A470T strains. Cells were grown in YPD to mid-log phase, and 100 μg of cycloheximide was added. Cells were incubated and collected at designated time point. The proteins were prepared, and Mre11 antibody was used to detect Mre11 and Mre11A470T protein. Mre11A470T has the same protein stability as Mre11. Actin was observed after stripping the blot and probing with an anti-actin antibody.
(TIF)

**S6 Fig. Mre11 RNA concentrations in genomic versus ectopic sites.** To test the transcriptional expression level of overall Mre11 in strains carrying two loci [genomic and ectopic], real time-qPCR of the reverse transcribed RNA was conducted as described in Materials and Methods. The first, second, and third bars refer to WT [g-MRE11] [as a control], e-MRE11 g-MRE11 and e-mre11A470T g-MRE11 strains, respectively. The expression of both e-MRE11 g-MTE11 and e-mre11A470T g-MRE11 were three- to four-fold greater than strains having only a single genomic locus. The Y axis represents the fold increase in transcripts relative to wild type.
(TIF)

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**Author Contributions**

**Conceptualization:** Arthur J. Lustig.
Data curation: In-Joon Baek, Daniel S. Moss, Arthur J. Lustig.

Formal analysis: In-Joon Baek, Daniel S. Moss, Arthur J. Lustig.

Funding acquisition: Arthur J. Lustig.

Investigation: In-Joon Baek, Daniel S. Moss, Arthur J. Lustig.

Methodology: In-Joon Baek, Daniel S. Moss.

Project administration: Arthur J. Lustig.

Resources: Daniel S. Moss, Arthur J. Lustig.

Software: In-Joon Baek, Arthur J. Lustig.

Supervision: Daniel S. Moss, Arthur J. Lustig.

Validation: In-Joon Baek, Daniel S. Moss.

Visualization: In-Joon Baek.

Writing – original draft: Arthur J. Lustig.

Writing – review & editing: In-Joon Baek, Daniel S. Moss, Arthur J. Lustig.

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