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Overcoming natural replication barriers: differential helicase requirements

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ABSTRACT

DNA sequences that form secondary structures or bind protein complexes are known barriers to replication and potential inducers of genome instability. In order to determine which helicases facilitate DNA replication across these barriers, we analyzed fork progression through them in wild-type and mutant yeast cells, using 2-dimensional gel-electrophoretic analysis of the replication intermediates. We show that the Srs2 protein facilitates replication of hairpin-forming CGG/CCG repeats and prevents chromosome fragility at the repeat, whereas it does not affect replication of G-quadruplex forming sequences or a protein-bound repeat. Srs2 helicase activity is required for hairpin unwinding and fork progression. Also, the PCNA binding domain of Srs2 is required for its *in vivo* role of replication through hairpins. In contrast, the absence of Sgs1 or Pif1 helicases did not inhibit replication through structural barriers, though Pif1 did facilitate replication of a telomeric protein barrier. Interestingly, replication through a protein barrier but not a DNA structure barrier was modulated by nucleotide pool levels, illuminating a different mechanism by which cells can regulate fork progression through protein-mediated stall sites. Our analyses reveal fundamental differences in the replication of DNA structural versus protein barriers, with *Srs2* helicase activity exclusively required for fork progression through hairpin structures.

INTRODUCTION

Replication does not proceed smoothly through genomes, but encounters multiple types of barriers that must be traversed. Two types of barriers that have been studied are sequences that form alternative DNA structures, and tightly bound proteins or protein complexes (1). Sequences that are known to form DNA structures and affect DNA replication are associated with genome instability and several human diseases (2,3). Thus, it is of pivotal interest to study the cellular strategies used for replication through these types of barriers. One of the strategies employed by the cell is the use of DNA helicases, specialized enzymes that use energy from adenosine triphosphate (ATP) hydrolysis to unwind DNA (4). Despite a wealth of data on helicase unwinding of DNA structures *in vitro*, little is known about which helicases act *in vivo* to facilitate replication through different types of structural barriers.

In addition to the conventional B-form double helix, DNA can form several alternative structures differing in their base pairing schemes, number of paired DNA strands, or both. Examples include intrastrand hairpins, G-quadruplex (G4) DNA and triplex DNA, all of which can interfere with DNA replication (1). Hairpin-forming trinucleotide repeat sequences such as CTG/CAG, CGG/CCG and triplex-forming GAA/TTC repeats stall or slow replication in yeast and humans (3,5,6). In addition, analysis of replication intermediates by two-dimensional gel electrophoresis (2D gels) showed that molecules migrating like reversed forks are formed during replication of a $(CAG/CTG)_{55}$ trinucleotide repeat tract on a yeast chromosome (7). Expansion of triplet repeat sequences is the cause of inherited human diseases including fragile X syndrome (FRAXA), myotonic dystrophy (DM1), Huntington's disease (HD), Friedreich's ataxia (FRDA) and many others, underlining the importance of studying replication of these sequence barriers (8). Structure-forming sequences such as expanded triplet repeats, triplex and inverted repeat sequences are also sites of chromosome fragility (9,10).

DNA sequences with G4 forming potential are another class of sequences that could potentially interfere with DNA replication. G4 DNA is abundant in the eukaryotic genome, especially at the rDNA loci, telomeres, mammalian immunoglobulin heavy chain class switch

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regions and at promoter regions (11–13). For most of these sequences it is not yet clear how frequently the G4 structure forms *in vivo* and in what circumstances, yet there is quite a bit of indirect evidence that G4 structures can and do form in cells (12). Similarly, although it is not clear whether G4 forming sequences can function as potent replication barriers *in vivo*, biochemical studies have shown their ability to block DNA polymerases *in vitro* (14). Accordingly, replication of G4 DNA sequences is thought to require specialized helicases (12). *In vitro* experiments have shown that the 5′–3′ helicase Pif1 (15) and the 3′–5′ helicase Sgs1 (16) and its human homolog BLM (17) can efficiently unwind G4 DNA. In addition, Pif1 deficiency leads to the instability of the G4 forming human CEB1 minisatellite (15), and fragility of naturally occurring G4 motifs in yeast (18).

Tightly bound protein complexes are another situation that can stall replication forks (1). Examples include the polar fork barrier caused by binding of the Fob1 protein at yeast rDNA, active transcription complexes at tRNA genes and protein–DNA complexes at centromeres (1). In some instances stalls have been associated with chromosome rearrangements and fragility (19,20). In budding yeast, the Rrm3 helicase is required for normal replication across various non-histone protein complexes (21), and the fork stabilizer protein Tof1 and its fission yeast homolog Swi1 are required for maintenance of programmed protein-mediated stalls (22–24). In contrast, absence of Tof1 leads to increased fork stalling at a CGG repeat barrier (5).

Telomeric DNA repeats, in addition to their structure-forming potential, are also bound by protein complexes that could potentially interfere with DNA replication. Budding yeast telomeric sequences are the target of the Rap1 protein, an abundant and essential protein (25). Even though yeast telomeric sequences can form G4 structures *in vitro*, their function as a fork barrier was shown to be due to binding of the Rap1 protein rather than due to G4 formation (26). Budding yeast Rap1 possesses highly sequence specific binding exclusively to yeast poly ($G_{1–3}T$) telomeric sequences and not to Oxytricha (G_4T_4), Tetrahymena (G_4T_2) or Dictyostelium ($C_{1–8}T$) telomeric sequences (27). The Rrm3 helicase, consistent with its role in promoting replication progression across protein barriers, was shown to promote replication fork progression through yeast telomeric and subtelomeric DNA bound by Rap1 (28). In fission yeast, the telomeric binding protein Taz1 is required for efficient replication across telomeric sequences (29)

We have recently found that both the Srs2 and Sgs1 helicases were important for preventing fragility and instability of an expanded CAG/CTG repeat sequence in budding yeast, and that replication through the repeat was altered in their absence (7). Sgs1, a member of the ubiquitous RecQ helicase family which includes human helicases mutated in Bloom's and Werner's syndromes, BLM and WRN, has roles in both replication fork maintenance and resolution of recombination intermediates (4). Srs2, a member of UvrD protein family, is a multifunctional protein having ssDNA-dependent ATPase, helicase and Rad51 dissociation activities (30). The absence of Srs2 is associated with unscheduled recombination, accumulation of toxic recombination intermediates, increased duplication mediated genome rearrangements and decreased double-stranded break (DSB) repair efficiency (30). It is not clear, which of the phenotypes that occur in cells lacking Srs2 are due to difficulties during replication. Based on the results with CAG repeats, one possibility is that the Srs2 protein facilitates replication of various structure-forming sequences. The Srs2 helicase has been shown to preferentially unwind CTG hairpin DNA *in vitro* (31) but has not been tested on other types sequences or fork barriers *in vivo*.

Because of their significance for genome stability, it is important to gain a better understanding of the factors that facilitate replication of DNA sequences that stall replication forks, and determine whether there are mechanistic differences between replication through various DNA structure and protein barriers. To that aim, we analyzed the replication of three kinds of DNA sequences—hairpin-forming repeats, G4-forming runs and protein-bound sequences—in yeast strains devoid of helicases that have been previously suggested to or been shown *in vitro* to be required for normal replication of these sequences. Here we report that in addition to its demonstrated role in stabilizing hairpin-forming CTG/CAG sequences (7), the Srs2 protein facilitates replication of hairpin-forming CGG/CCG sequences via its helicase activity, and prevents CGG/CCG repeats from undergoing frequent breakage. Other tested helicases could not facilitate CGG hairpin replication, however Pif1 and Rrm3 were required to bypass a polar protein barrier at the yeast telomere sequence. We also discovered contrasting effects of nucleotide pool levels on structure versus protein barriers. Thus our analyses reveal fundamental differences between replication of sequence and protein barriers in eukaryotes.

MATERIALS AND METHODS

Plasmid constructs

YEp24 plasmids containing 40 repeats of CGG and CCG have been previously described (32). G_4T_4 sequences were derived from the previously described pVS20 plasmid (33). pVS20 was digested with NsiI/SmaI to release a 159 bp fragment that also contained 13 repeats of G_4T_4/C_4A_4 sequence. This fragment was blunted by Klenow reaction and ligated in both orientations into the AseI site of YEp24 plasmid. The orientation and the sequence lengths of the inserts were confirmed by PCR and sequencing. Human telomeric sequences $(TTAGGG/AATCCC)_{12}$ and synthetic yeast telomeric sequences Ytel $(TGTGTGGG/ACACACCC)_{15}$ were originally cloned by Sergei Mirkin into the SmaI site of pUC19. These sequences were released from pUC19 by EcoRI/HindIII digestion, blunted by Klenow and ligated in both orientations into the AseI site of YEp24. Inserts were verified by PCR and sequencing. All the sequence files are available on request.

Strains

All strains share the isogenic BY4705 background. Deletions were made by the standard PCR deletion method using the pFAKANMX6 cassette or in the case of *pif1Δ* by pFATRP (34,35). The various YEp24 plasmids were transformed into either wild-type (WT) or mutant strains using standard yeast transformation procedures and selected on media lacking uracil. The length of repeat tracts were confirmed by yeast colony PCR as described in (7) with primers flanking the inserts, and amplicons were resolved on 2% metaphor gels.

2-dimensional gel analysis

Replication intermediates were isolated by the method described in Huberman *et al*. (36) with the following modifications. Glass bead method of cell disruption was replaced with the spheroplasting method [described in (37)]. DNA after restriction digestion was isolated by Fermentas 'gene-jet' gel extraction kit and eluted in 25 µl of TE before loading with 1× bromophenol blue into the first dimension gels. First dimension 0.9% agarose gels were run in 1× TBE buffer for 16 h at 1 v/cm; second dimension 0.4% agarose gels containing 0.3 µg/ml ethidium bromide were run at room temperature for 24 h at 2 v/cm. Southern transfers were done by standard procedures using X-bond membrane (GE Life Sciences) and probed with ^{32}P-labeled (Fermentas Decalabel/ Stratagene Prime-it II) 630 bp fragment obtained by PCR from YEp24 plasmid comprising nucleotide positions 6952–7581.

Quantification of stalls

Semi-quantitative analysis of 2D gels was done using BioRad Pharos FX PhosphorImager using Quantity One software. The percentage of the stalled intermediates was calculated from the 2D profile as follows. Using the volume tool provided by the Quantity One Software, a contour was traced along the border of pause signal and designated the symbol 'a' (pause signal = a). The total Y intermediates was counted by the drawing along the contour of the Y arc and designated the symbol 'b' (total Y replication intermediates = b). The percentage of stalled intermediates was then calculated as [(a/b) × 100]. Quantification of the peak intensities was done similar to the methods described in Voineagu *et al*. (38) with the following modifications. The Microsoft Excel data sheet containing the radioactive counts were exported to GraphPad Prism Software and the baseline was normalized using the in-built 'baseline removal' feature. Statistics: Graphpad Prism software was used to calculate mean percentage stalled intermediates. Mean percentage stalled intermediates were compared by unpaired *t*-tests.

Fragility assay

The fragility assay was done essentially the same way as described by Balakumaran *et al*. (39), except that prior to conducting the fragility assays the CGG/CCG tract lengths were determined PCR amplification and sizing on a 2% Metaphor agarose gel. PCR reaction conditions were: 1× IDpol buffer without $MgSO_4$, 2 mM $MgSO_4$, 1× CG buffer (ID labs), 0.3 µM of each primer, 200 µM dNTPs and 0.4 µl of Taq polymerase (per 12.5 µl reaction mixture; unknown units; Phoenix Lab, Tufts University). The following amplification parameters were followed: $(95^{\circ}C^{5.00})_1$ $(95^{\circ}C^{0.30}$, $54^{\circ}C^{1.00}$ $72^{\circ}C^{4.00})_{35}$ $(72^{\circ}C^{7.00})_1$. Primer pairs caggctgggaagcatatttgagaagatgc and attcaaagacgtagcaacaacaacacgagca were used to amplify CGG repeats and primer pairs agaaagactagcttcttttcgggtgatgt and aagcatatttgagaagatgcggccagc were used to amplify CCG repeats. The breakage rates were determined by the method of Maximum Likelihood using the SALVADOR program (40).

DNA substrates for helicase assays

The DNA substrates harboring no hairpin, $(CGG)_{11}$ triplet or $(CTG)_{11}$ triplet were obtained by hybridizing 5′-radiolabeled oligonucleotides H1 (5′-GTGTAGCACC GTGGTTTAGGCTGGCACGGTCG-3′) to H2 (5′-CG ACCGTGCCAGCCTAAACCACGGTGCTACACTTG CCCGTTTTATT-3′), H3 (5′-CGACCGTGCCAGCCTA AACCA$(CGG)_{11}$TGCTACACTTGCCCGTTTTATT-3′) or H4 (5′-CGACCGTGCCAGCCTAAACCA$(CTG)_{11}$T GCTACACTTGCCCGTTTTATT-3′). The labeling of H1 was done with (γ-^{32}P) ATP and T4 polynucleotide kinase and the labeled oligonucleotide was annealed to partner oligonucleotides by heating an equimolar amount of the oligonucleotides at 95°C for 10 min, followed by slow cooling to room temperature. The substrates were separated from unannealed oligonucleotides in a 10% non-denaturing polyacrylamide gel run in TAE buffer (30 mM Tris-acetate, pH 7.4, 0.5 mM EDTA) and recovered from the gel by electro-elution in a dialysis tubing into TBE buffer (100 mM Tris borate, pH 8.0 and 2.0 mM EDTA) at 4°C.

Helicase assays

Srs2 and Sgs1 were purified as described before (41,42). For helicase assays, ^{32}P-labeled DNA substrates (5 nM) were incubated with either Srs2 (5 nM) or Sgs1 (0.25 nM), which gives an equivalent level of unwinding of the non-hairpin substrate, in 60 µl reaction buffer (40 mM Tris–HCl, pH 7.5, 50 mM KCl, 1 mM DTT, 100 µg/ml BSA, 2 mM $MgCl_2$, 2 mM ATP and the ATP regenerating system consisting of 20 mM creatine phosphate and 20 µg/ml creatine kinase) at 30°C. Aliquots of the reactions were taken at the indicated times and treated with SDS (0.2% final) and proteinase K (0.5 mg/ml) for 3 min at 37°C. A 10-fold molar excess of unlabeled H1 oligonucleotide was added to prevent reannealing of the products. The reaction mixtures were run a 10% polyacrylamide gel in TBE buffer at 4°C, and the gel was dried onto DE3 paper and then subject to phosphorimaging analysis.

Hydroxyurea experiment

A 400 ml culture of yeast cells containing YEp24 plasmids was grown in selective media to an OD of ~0.4. Hydroxyurea (SIGMA) was added to the culture to a final concentration of 0.2 M. Cells were monitored for

their growth by OD measurements at 600 nm. When the cell culture OD reached an OD of 0.8–1.0, cells were harvested and analyzed for the strength of the stall.

RESULTS

The Srs2 helicase facilitates replication of CGG/CCG repeats *in vivo*

Since the Srs2 helicase plays a role in replicating across hairpin-forming CTG repeats (7), we investigated whether it was important for replication of other types of DNA structure or protein barriers. In order to determine whether Srs2 facilitates replication of CGG/CCG repeats *in vivo,* we used 2D gels to visualize replication intermediates and thereby analyze replication fork progression across 40 repeats of CGG/CCG sequences in the presence and absence of Srs2 helicase. Either due to their propensity to form hairpins or G4 structures, 40 repeats of CGG profoundly stall replication *in vivo* in all model systems studied including bacteria, yeast and mammalian cells (5,32,43) and thus are one of the strongest known DNA structure barriers.

CGG/CCG repeats were cloned in the multicopy 2-micron YEp24 plasmid in two orientations, such that CGG repeats were replicated as either the lagging strand or leading strand template (Figure 1; the sequence nomenclature refers to the repeat on the lagging strand template). Stalling of replication, due to the accumulation of one species of Y shaped fork intermediates, can be visualized as bulge along the smooth Y arc. Replication of the control YEp24 plasmid in either WT or *srs2Δ* cells showed no significant sites of stalling (Supplementary Figure S1). As was previously shown (32), 40 repeats of CGG sequences visibly stalled replication in WT yeast cells in both orientations (Figure 2a). The stalled intermediates represented an average of 5.2 and 4.5% of the total Y-shaped replication intermediates for the CGG or CCG orientations respectively. In a Srs2 deficient background, the stall signal increased to 12.5% and 11% for CGG and CCG orientations, a 2.5-fold increase over WT, indicating that in the absence of the Srs2 helicase CGG/CCG repeats become more profound barriers to replication progression. These results show that the Srs2 helicase is required for efficient fork progression through CGG/CCG repeats.

CGG repeats could potentially form either hairpin or G4 structures (3) and G4 forming sequences have been hypothesized to stall replication forks, requiring specialized helicases to facilitate replication across them (12). Therefore, we tested whether the Srs2 helicase was required to facilitate replication across sequences with the potential to form G4 but not hairpin structures, the *Oxytricha* $(G_4T_4/C_4A_4)_{13}$ or human telomeric sequences $(T_2AG_3/A_2TC_3)_{12}$ (Oxytel and Htel, respectively). 2D gels showed accumulation of Y intermediates at both Oxytel and Htel sequences, indicating that these sequences can act as fork barriers (Figure 2b and c). The percentage stall signals were 7.5 and 3.3% for Oxytel and Htel sequences, respectively when the G-rich strand formed the lagging strand template, which is consistent with the demonstrated greater stability of G_4T_4 compared to G_3T_2A G4 structures (44), and suggests that these structures are forming *in vivo* (Figure 2b and c). We also observed fork stalling when the C-rich strand formed the lagging strand template, which is opposite to the orientation found naturally at telomeres (6.8 and 4.2% stall signals for C_4A_4 and Htel-opp (A_2TC_3) respectively). Quantification of the amount of pause signal in the absence of Srs2 with Oxytel or Htel sequences showed no significant increase in stalled intermediates compared to WT for either orientation. Therefore, the Srs2 helicase does not appear to play a role in unwinding G4 structures.

Escherichia coli homologs of *Srs2*, UvrD, RepD and PcrA helicases, can aid in replication across DNA–protein complexes (45), and Srs2 can dislodge the Rad51 protein from DNA (41,46). In order to determine whether Srs2 facilitates replication across a protein barrier, a tract of the yeast telomeric sequence, which binds the Rap1 protein with strong affinity, was analyzed. Based on comparisons between the pause strength of *Tetrahymena* and yeast telomeric sequences in yeast and the known affinity

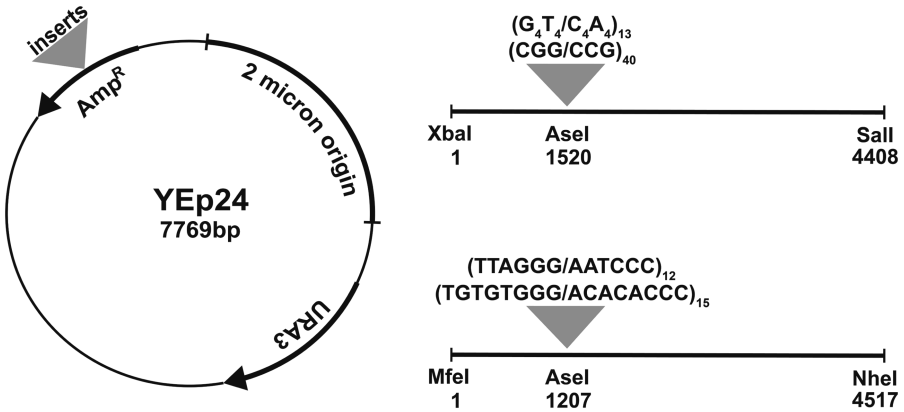

Figure 1. Schematic of the YEp24 construct and the location of the insert sequences. Indicated sequences were cloned into the AseI site of YEp24 in both orientations. YEp24 plasmids containing either $(CGG/CCG)_{40}$ or (G_4T_4/C_4A_4) were digested with the restriction enzymes SalI and XbaI, generating a fragment of 4.4 kb. YEp24 plasmids containing either Htel or Ytel sequences were digested with MfeI and NheI, generating a fragment of 4.5 kb.

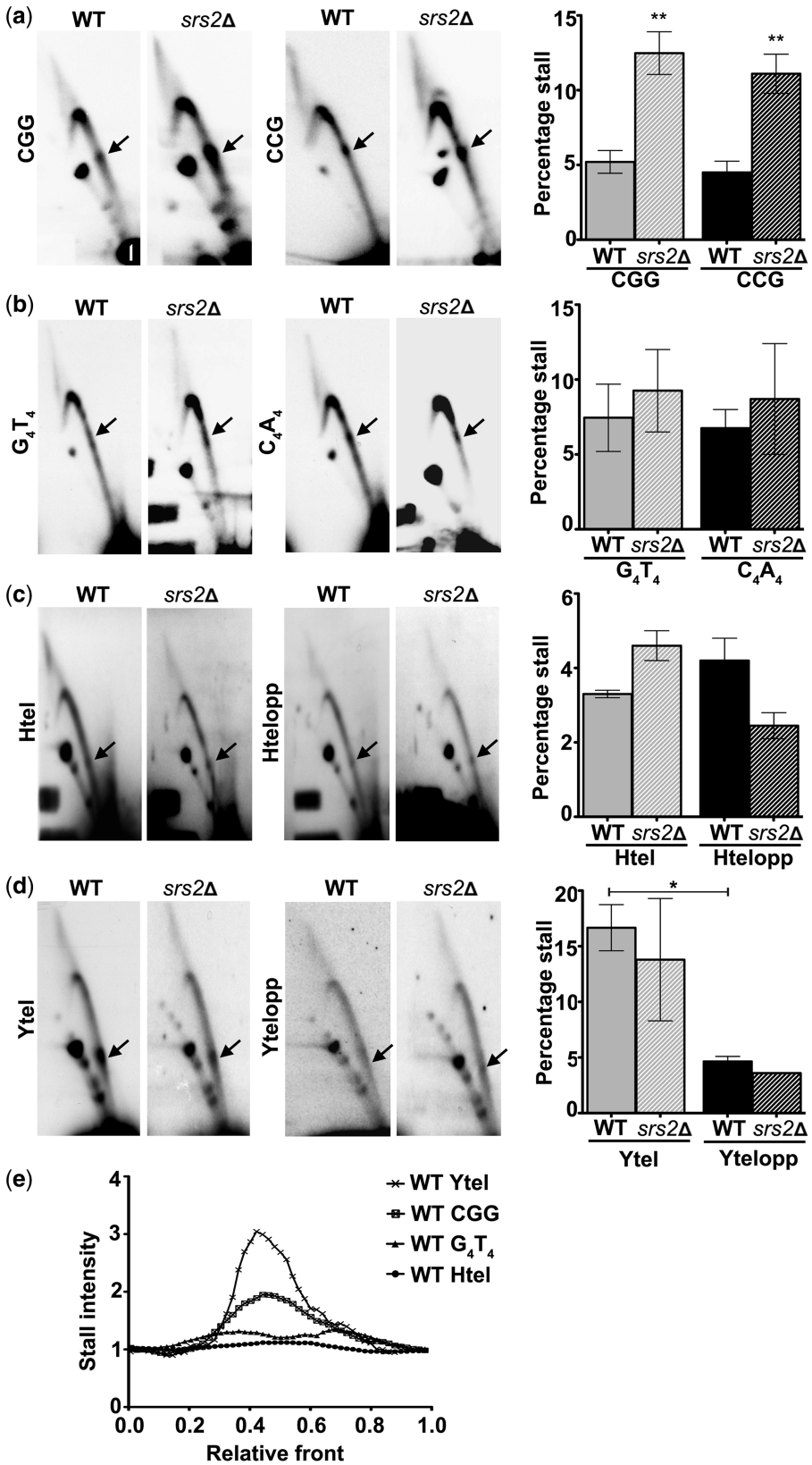

Figure 2. Analysis of replication through sequence and protein barriers in WT and *srs2Δ* cells. Replication of (**a**) CGG and CCG repeats, (**b**) Oxytel sequence, (**c**) Htel sequence, (**d**) Ytel sequence. Sequence nomenclature refers to the lagging strand template. Solid arrows indicate location of the stall due to the insert. Quantification of the percentage of stalled intermediates compared to the Y arc signal is shown in the graphs at right. (**e**) Densitogram showing relative peak intensities of the stall by CGG, Oxytel, Htel and Ytel sequences with G rich sequences on the lagging strand template. The following number of experiments were done for each strain: WT CGG, 7; WT CCG, 4; *srs2Δ* CGG, 5; *srs2Δ* CCG, 4; WT Ytel, 3; all others, 2. Error bars indicate standard error of the mean (SEM). Stars indicate a significant difference between the wild-type and mutants (Student's *t*-test, $^{**}P \leq 0.01$; $^{*}P \leq 0.05$).

of the *Rap1* protein to TG_{1-3} DNA, Makovets *et al.* (26) concluded that the stall at yeast telomeric DNA was due to a Rap1 protein complex rather than G4 formation. Consistent with this conclusion, the yeast telomeric sequences severely hampered progression of replication in WT cells (17% stall signals, Figure 2d). Notably, in the opposite orientation, when the lagging strand machinery replicated the C rich strand, the amount of pause signals was significantly reduced by 3.6-fold to 4.7% indicating an orientation dependence of the stall. This orientation dependence of the stall could be due to two non-exclusive scenarios; (i) preferential formation of secondary structure in one orientation (ii) asymmetric binding of protein complexes to their cognate sequences forming polar fork barriers. Based on the experiments by Makovets *et al.* (26) and by our data that G_4T_4 and C_4A_4 showed no difference in pausing strength, it is most probable that the orientation dependence is due to asymmetric binding of a protein complex. The Rap1 protein is an excellent candidate, as it has been shown previously to interact with Ytel sequences *in vivo* and it is abundant at yeast telosomes (47,48). Unfortunately, lowering the expression of Rap1 also affected the overall efficiency of replication (Supplementary Figure S2) precluding a definitive test of this hypothesis. Quantification of the stalled intermediates in the absence of the Srs2 helicase revealed no significant difference compared to the WT, and the orientation dependence was still observed (Figure 2d), indicating that the Srs2 helicase is not required for replication forks to progress past telomeric protein–DNA complexes. Based on the 2D gel data presented here, along with results with CTG repeats (7), we conclude that the Srs2 helicase facilitates replication exclusively through hairpin-forming sequences and is not involved in replication through G4 structures or protein barriers. These data also suggest that the CGG/CCG sequences are forming hairpins rather than G-quadruplexes *in vivo*, since they have different replication requirements.

Comparison of the peak intensities of the stalls (rather than percent stall) induced by the various sequences revealed several interesting points. The stalls due to the G4 DNA forming G_4T_4 and G_3T_2A sequences were more spread out and diffuse in quality, than other stalls, sometimes appearing as two stalls, and therefore had a lower peak intensity compared to the CGG repeats (Figure 2e). The Ytel protein-mediated stall was by far the strongest with a peak intensity >3-fold over background. Moreover, even though the G richness of G_4T_2 and CGG sequences are identical (~67%) the former showed a less intense stall than the latter. In summary, the propensity to stall replication for the sequences tested here is: (Ytel) protein stall > CGG hairpin > G4 forming sequence.

Srs2 helicase prevents breakage of hairpin-forming CGG/CCG repeats

Expanded CGG/CCG repeats have been characterized as regions susceptible to chromosome fragility at two human X chromosomal loci, FRAXA and FRAXE as well as on a yeast chromosome (49,50). In lymphocytes, the fragile sites express when cells are grown in media containing anti-folate metabolites that slow down replication, and CGG fragility is also modestly increased by depletion of dNTP pools by hydroxyurea (HU) in yeast (50). Since replication of CGG/CCG repeats was negatively affected in cells deficient in Srs2 helicase, we asked whether the replication defect is translated into a fragility phenotype. We determined the rate of breakage of a CGG/CCG containing chromosome and a no repeat control using a previously designed chromosome breakage assay (50) (Figure 3a). Breakage at the CGG repeats induces direct repeat recombination between the *LYS2* duplication, resulting in loss of the *URA3* gene and cellular resistance to the drug 5-fluoro-orotic acid (5-FOA) (50,51). Fluctuation analysis was used to measure the rate of recombination in cells with a $(CGG)_{81}$ or $(CCG)_{81}$ tract as well as in a corresponding no tract control strain. Similar to the results previously obtained by Balakumaran *et al.* (50), WT cells with an expanded repeat tract showed an increased rate of recombination compared to the no tract control: a 3.5-fold increase for the CGG orientation and a 2.1-fold increase for the CCG orientation (Figure 3b). In the absence of *SRS2*, the rate increased dramatically: 17.4 fold for $(CGG)_{81}$ compared to the *srs2*Δ no tract control and 20.1-fold compared to the WT CGG strain. In the absence of *Srs2* helicase and in the CCG orientation, the 5-FOA resistance rate was increased 2.1-fold over the *srs2*Δ no tract control and 3.5-fold over the WT CCG strain. Thus, our results show that the fragility of a CGG/CCG containing chromosome is dramatically increased in the absence of the Srs2 helicase, especially when the CGG repeats are on the lagging strand template. The orientation dependence of fragility in the absence of Srs2 is interesting given that the amount of stalled intermediates did not significantly differ between CGG and CCG repeats (Figure 2a).

Srs2 facilitates replication through a CGG hairpin via helicase unwinding activity and PCNA interaction

The Srs2 protein has both helicase activity and single-stranded DNA translocase activity, both of which are ATP-dependent (30). It also can displace Rad51 from a presynaptic filament, an activity which is dependent on interaction with Rad51 and which is needed for the anti-recombinase function of the Srs2 protein (41,46,52). To gain a mechanistic understanding of how the Srs2 protein facilitates replication of the CGG repeat, we utilized the well-characterized K41R mutant of Srs2. The K41R mutant has lost the ability to hydrolyze ATP, and thus both helicase and translocase activities (30). When we quantified the amount of stalling in the K41R mutant, it was equivalent to the *srs2*Δ strain, indicating that either DNA unwinding or translocation was necessary to bypass the hairpin structure (Figure 4a). It remained possible that Rad51 displacement (30) was the important function. To compromise Rad51 displacement without affecting the helicase activity, we utilized an Srs2 mutant that is missing residues 875–902, which encompasses most of the defined Rad51 interaction domain, and which has been shown to be largely deficient in Rad51 displacement and anti-recombinase activity but

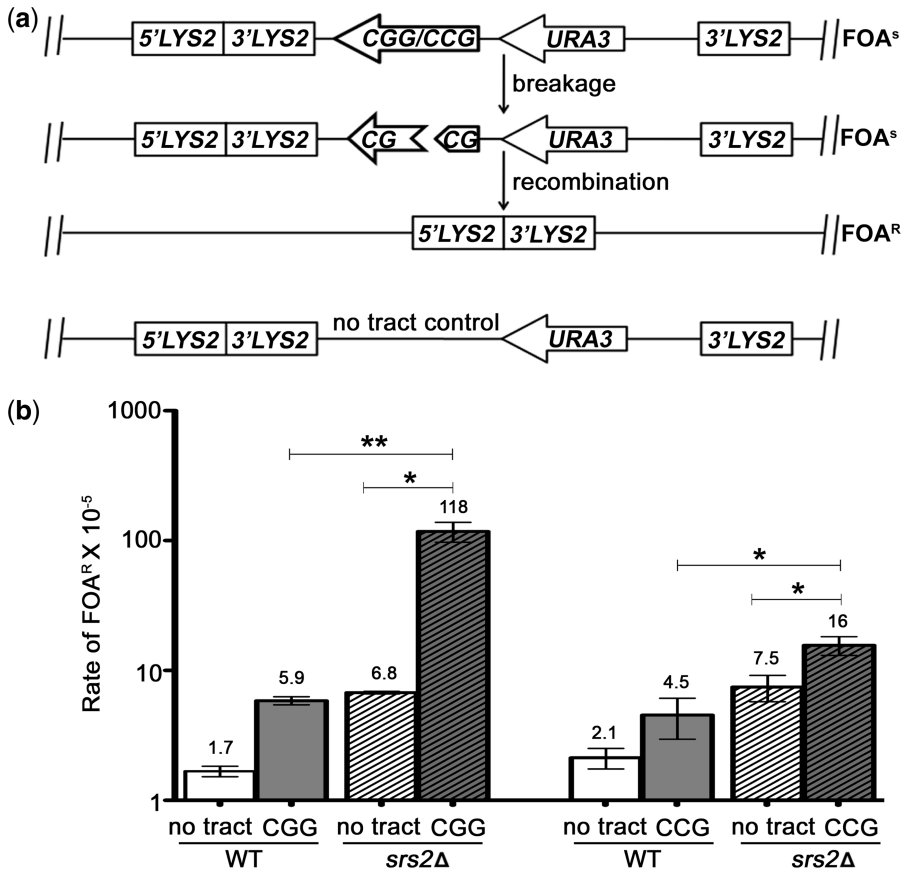

Figure 3. Direct repeat recombination assay to measure the breakage rate of CGG and CCG repeats in WT and srs2*Δ* cells. (**a**) Schematic of the genetic assay. The construct contained 81 repeats of CGG in either orientation and the *URA3* gene, integrated at the *LYS2* locus on yeast chromosome II such that they are flanked by 708 bp of duplicated *LYS2* sequence. Recombination between the *LYS2* duplication will result in loss of the *URA3* gene and resistance to the drug 5-FOA. (**b**) Mean rates (shown on top of the bars) ± SEM were compared by a pooled variance *t*-test. Asterisks indicate significant difference between the indicated categories. $^{**}P \leq 0.01$, $^{*}P \leq 0.05$.

yet retains WT levels of ATPase and helicase activity (52). The Δ875–902 mutant was competent for replication through the CGG repeat, with the amount of stalling equivalent to WT (Figure 4a), providing evidence that Rad51 displacement is not the primary Srs2 activity needed for CGG repeat bypass. We conclude from this data that the Srs2 helicase activity is essential for replication past a CGG barrier, most likely through unwinding of the hairpin structure.

To directly test for CGG hairpin unwinding, a substrate was created that contained a $(CGG)_{11}$ hairpin and incubated with purified Srs2 protein. Unwinding of the CGG hairpin substrate by Srs2 was about 3-fold less efficient than a control substrate, consistent with the data that the CGG repeat is a replication barrier *in vivo* (Figure 4b). Nonetheless, the Srs2 protein was able to unwind the CGG hairpin substrate at a rate comparable to the less stable CTG hairpin, indicating that the G-G basepairs do not additionally impede unwinding. Interestingly, full length Sgs1, which is a potent helicase (53), was less efficient in unwinding the CGG hairpin when compared to the control substrate, e.g. Srs2 showed 2-fold more unwound product at 5 min (Figure 4b and c). A previous study comparing unwinding of an equivalent $(CTG)_{11}$ substrate by Srs2 and utilizing a truncated, less active form of Sgs1 found a 3- to 4-fold kinetic advantage for Srs2 (31). Nonetheless, despite the kinetic advantage of Srs2, Sgs1 can also unwind the $(CGG)_n$ hairpin substrates. The fairly subtle unwinding advantage of the CGG hairpin by Srs2 observed *in vitro* did not seem to account for the much more profound difference in the unwinding effect of the two helicases observed *in vivo*.

Based on the above data, we hypothesized that the *in vivo* ability of the Srs2 helicase compared to the Sgs1 helicase to facilitate CGG replication could be due to their differential locations or interactions at the fork, with substrate specificity playing a lesser role. The C-terminus of Srs2 has been shown to interact with sumoylated PCNA, and this interaction is important for its localization to the replication fork (54–56). Thus, interaction with PCNA could potentially position Srs2 to unwind hairpins during replication. To test this idea, we utilized a mutant missing the last 176 residues of Srs2, retaining residues 1-998, that has been shown to be deficient in PCNA interaction but retains both helicase activity and interaction with Rad51 (52,54,57). Remarkably, the percentage of forks stalled at the CGG sequence in this

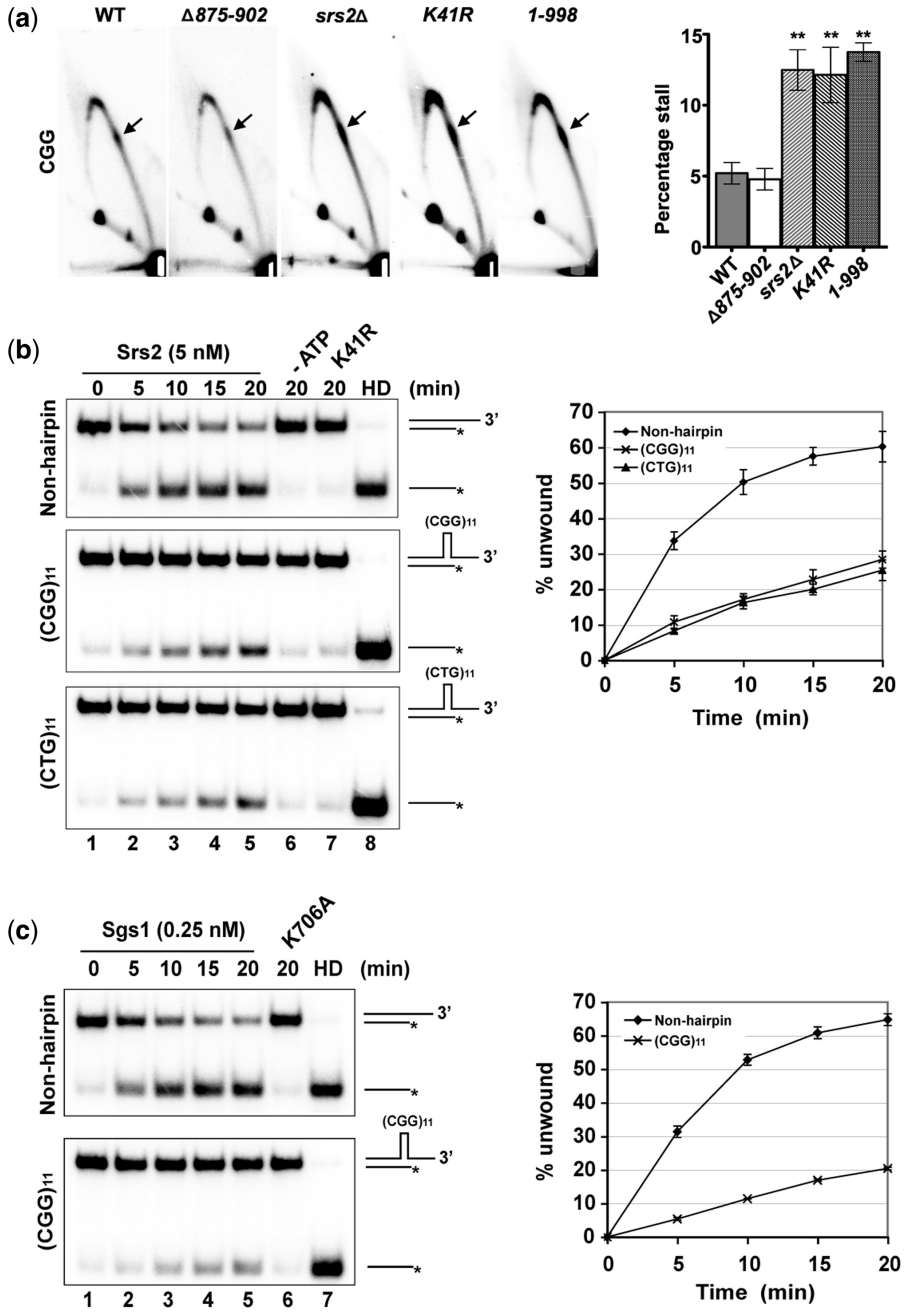

Figure 4. Determination of the Srs2 activity needed for fork progression past a CGG barrier and unwinding efficiencies. (**a**) Analysis of replication intermediates was performed and quantified as in Figure 2a. Percentage stall is the average of 4, 3, and 2 experiments for Δ875-902, K41R and 1-998, respectively, ± SEM. WT and *srs2*Δ values as in Figure 2a. (**b**) Srs2-mediated unwinding of substrates with no hairpin or a $(CGG)_{11}$ or $(CTG)_{11}$ triplet is shown. ATP was omitted in lane 6 and the helicase defective srs2-K41R mutant was examined in lane 7. Heat-denatured substrate (HD) was analyzed in lane 8. The mean values ±SD from three independent experiments of analyzing wild-type Srs2 activity are plotted on the graph. (**c**) Unwinding of the same substrates by the Sgs1 helicase. The helicase defective sgs1-K706A mutant was examined in lane 6, and heat-denatured substrate (HD) was analyzed in lane 7. The mean values ±SD from three independent experiments of analyzing wild-type Sgs1 activity are plotted.

mutant was the same as a strain deleted for Srs2. Thus, the Srs2–PCNA interaction is absolutely required for its ability to facilitate replication through a DNA hairpin.

Sgs1 and *Pif1* helicases are dispensable for replication through hairpin or G4 structures

Next we wished to determine the role of Sgs1 and Pif1 helicases in replication across DNA structure or protein barriers. Yeast Sgs1 and its human homologs WRN and BLM have been shown to unwind secondary structures formed due to G-G pairing *in vitro* (16,58,59). Moreover, Hershman *et al.* (60) found that yeast genes that can potentially form G-quadruplexes are selectively downregulated in *Sgs1* mutants. If the Sgs1 helicase is needed to unwind G-rich structures at the replication fork, an increase in fork stalling should be observed in

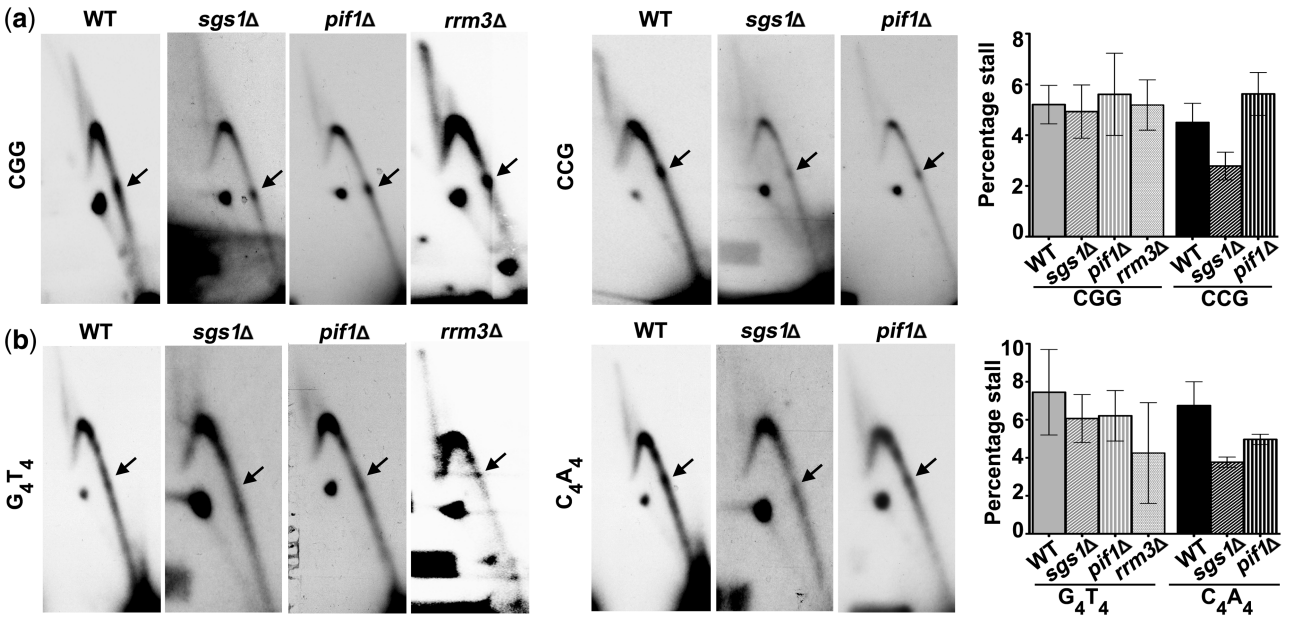

Figure 5. Analysis of replication through hairpin and G4 forming sequences in *sgs1Δ*, *pif1Δ* and *rrm3Δ* cells. Labels and symbols as in Figure 2. Percent stall is the average of at least two experiments ± SEM.

the absence of Sgs1. Notably, we could not detect any increase in the amount pausing at the CGG repeats, suggesting that secondary structures formed by CGG repeats can be replicated efficiently in the absence of Sgs1 (Figure 5a). This was also true for the opposite orientation when CCG repeats were replicated by the lagging strand machinery. In addition, we detected no increase in pausing when G_4T_4/C_4A_4 sequences were replicated in either orientation, indicating that replication of these sequences proceeded with normal efficiency in the absence of Sgs1 (Figure 5b). In fact, when the C-rich repeat was on the lagging strand template (CCG and C_4A_4), pausing looked uniformly decreased in the *sgs1*Δ strain, although these decreases were not large enough to be statistically significant. Therefore, the presence of Sgs1 may even facilitate structure formation or pausing of replication at structures in some situations.

The Pif1 helicase was shown to unwind G4 forming DNA sequences *in vitro*, and in its absence the G4 DNA-forming human CEB1 minisatellite was destabilized (15). More recently, it was shown to bind to a subset of G4 motifs in the yeast genome, and those motifs stimulated recombination and were more likely to mutate in a manner predicted to prevent G4 structure in *pif1* mutants (18). We hypothesized that if the Pif1 helicase helps replication proceed through G4 sequences, we should see an increase in the amount of pause signals in cells deficient in Pif1. However we did not detect any differences in the pause signals between WT and *pif1Δ* cells when either CGG/CCG or G_4T_4/C_4A_4 sequences where replicated in both orientations (Figure 5a and b). To test whether Sgs1 and Pif1 might each be compensating for the loss of the other, we also analyzed replication through the CGG repeat in the *sgs1Δ pif1Δ* double mutant. Again, there was no increase in fork stalling, but rather a decrease in the pause signal to a barely detectable level (Supplementary Figure S3). In summary, the lack of effect on fork pausing in *sgs1Δ* and *pif1Δ* mutants argues against a direct role for Pif1 or Sgs1 helicases in replication across hairpins or G-quadruplexes *in vivo*. Quite the opposite, there may even be a role for these helicases in facilitating formation of the structures, which then interfere with fork progression, or maintenance of the paused replication complex.

Replication through a telomeric DNA protein barrier is facilitated by Rrm3 and Pif1 helicases and by low nucleotide pools

Rrm3 helicase has been shown to be a 'sweepase' of non-histone protein–DNA complexes, aiding replication of telomeric and subtelomeric DNA (21,61,62). We could not detect differences in the replication profile of the hairpin-forming CGG repeats or quadruplex-forming G_4T_4 sequences in Rrm3 deficient cells (Figure 5) indicating that this helicase does not affect replication through DNA structures. However, the Ytel sequences showed a significant 2-fold increase in the stall signal as well as an altered pattern of replication characterized by an increase in aberrant joint molecules in the absence of Rrm3 (Figure 6). This is consistent with its known role in promoting replication through protein barriers. Furthermore, replication of Ytel sequences in the absence of Pif1 helicase also resulted in significantly increased stalling (1.6-fold, Figure 6), indicating that both Rrm3 and Pif1 facilitate replication past a telomeric protein barrier, though they have opposite polarities and effects on the rDNA protein barrier (61). In contrast, absence of the Sgs1 protein had no effect on the Ytel stall.

To further characterize the mechanistic differences between DNA structure and protein stalls, we treated

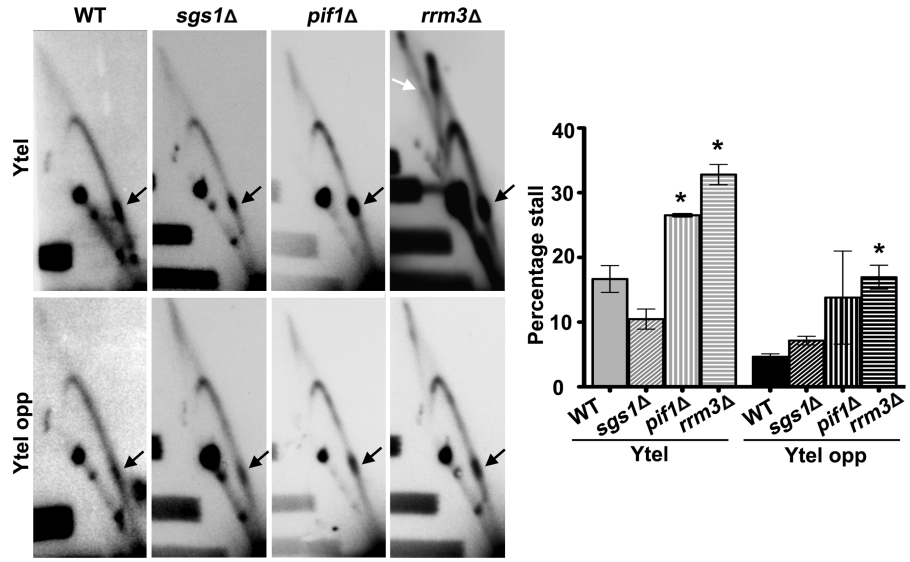

Figure 6. Analysis of replication through a telomere protein barrier in *sgs1Δ*, *pif1Δ* and *rrm3Δ* cells. Labels and symbols as in Figure 2. Percent stall is the average of at least two experiments ± SEM. White arrow indicates presumptive recombination structures.

cells with HU, which slows down replication due to its inhibitory effect on ribonucleotide reductase, lowering the dNTP pool inside the yeast cell. Cells were grown for approximately one division in the presence of 0.2 M HU and analyzed for the strength of the pause signal. Previous experiments from other laboratories have shown that in the presence of 0.2 M HU, polymerase epsilon is found 3.5–4 kb away from early firing origins, suggesting that forks can travel some distance from the origin of replication in these conditions (63). At this same concentration of HU, budding yeast cells are able to replicate their DNA, although at a considerably slower rate (64). Analysis of replication intermediates showed that Y arcs were clearly visible, indicating that forks originating from the early firing 2 micron origin 1.5 kb away are able to traverse the length of the 4 kb restriction fragment analyzed during the course of the experiment (Figure 7). Quantification of the CGG stall showed that the percent stall was no different from that of control cells grown in the absence of HU (control CGG mean = 5.2%, HU treated CGG mean = 5.0%) (Figure 7a). Therefore, we conclude that slowing down replication did not facilitate or further inhibit replication through a hairpin-induced stall.

We then analyzed the replication of YtelI sequences in the presence of HU. In contrast to the structure-mediated stall, HU significantly weakened the overall amount of pausing at the YtelI protein-mediated stall, (3.5% with HU compared to 17% without HU) (Figure 7b). The differing response to HU reveals a fundamental difference between sequence and protein barriers to replication. Several studies in both budding and fission yeast have shown a requirement for Tof1 (or its fission yeast homolog Swi1) for some (but not all) protein-mediated stalls, thus it was possible that a reduction of Tof1 occupancy at the stall in the presence of HU could explain the decreased stalling (22–24). 2D analysis of YtelI sequences in the absence of Tof1 resulted in a large reduction in the amount of pause signals (4.7% in *tof1Δ* compared to 17% in WT), indicating that Tof1 is also required at a telomere protein stall (Figure 7b). Strikingly, the combination of the *tof1Δ* and the presence of hydroxyurea completely eliminated the YtelI stall (Figure 7b). Thus, the reduction of stalling induced by the presence of HU or the absence of Tof1 occurs by two independent mechanisms.

Since we saw a reduction in the protein-mediated stall in the presence of reduced dNTP pools, we tested whether an increase in dNTP pools would result in an increase in the pause signal. In the absence of the yeast Sml1 protein, production of nucleotides is deregulated, resulting in increased dNTP pools (65). The amount of YtelI stall signal was increased 1.6-fold in the *sml1Δ* strain compared to that of WT (Figure 7b). We conclude that nucleotide pool levels are a potential modulator of telomere protein-mediated fork stalling.

DISCUSSION

Barriers to replication are potential inducers of genome instability. Here, we have analyzed how different fork barriers are replicated *in vivo*, directly comparing DNA structure versus protein barriers and the role of various helicases in their bypass. We have made use of sequences that have a strong tendency to form either hairpins or G-quadruplexes, or are known targets of protein complexes, to understand how these barriers are overcome by the replication machinery.

As reported previously (32), analysis of hairpin-forming triplet CGG/CCG repeat sequences revealed them to be strong barriers to replication. Absence of Srs2, a helicase that was previously shown to efficiently unwind short CTG hairpin structures *in vitro* (31) and to facilitate replication of long hairpin-forming CAG/CTG repeat sequences *in vivo* preventing their instability (7), was found

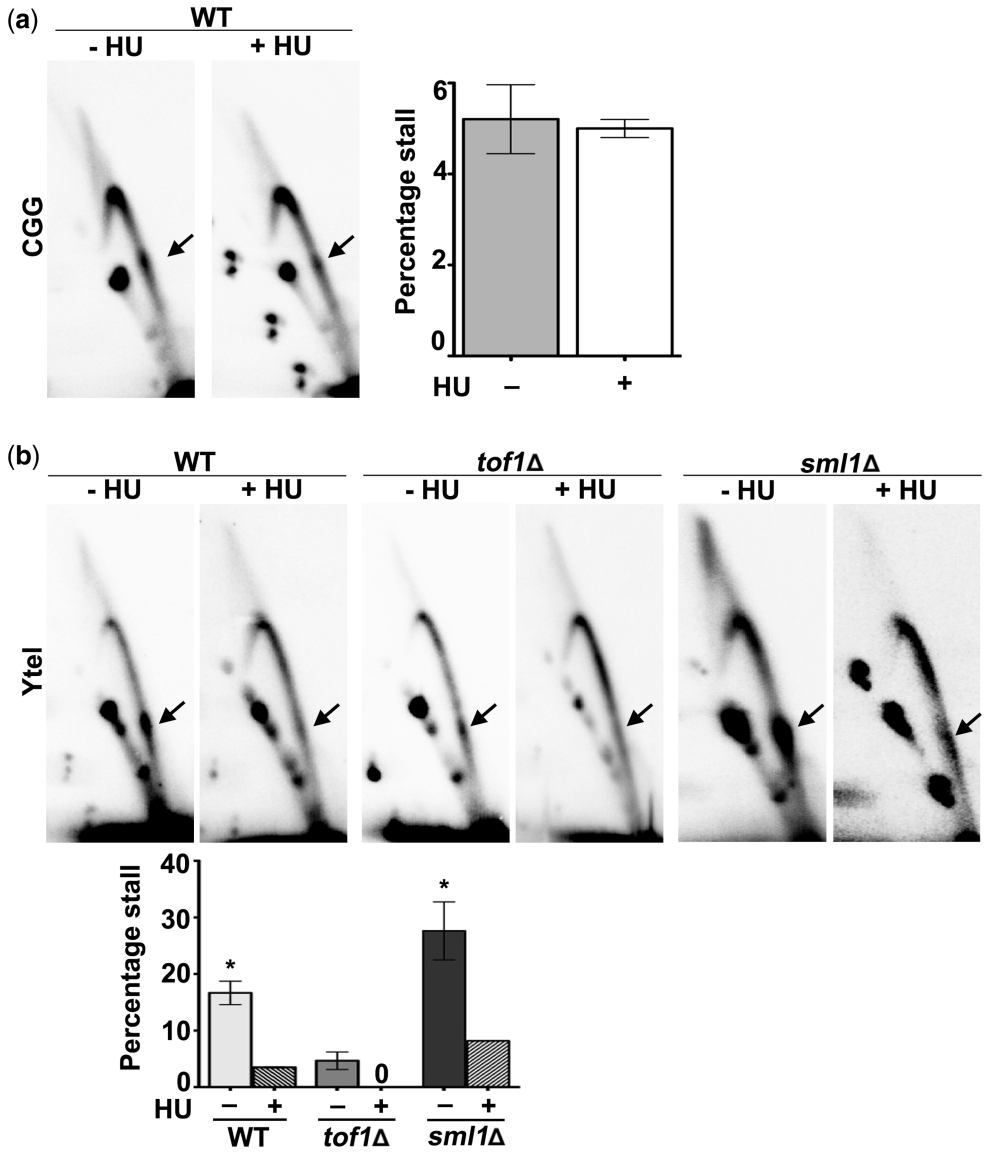

Figure 7. Effect of HU on replication of CGG and Ytel sequences. (**a**) Replication of CGG sequences in WT cells in the presence (+) and absence (−) of 0.2 M HU. Percent stall is the average of four experiments ± SEM. (**b**) Replication of Ytel sequences in the presence and absence of 0.2 M HU in WT and mutant cells. Percent stall is the average of at least two experiments ± SEM. Comparisons were made to −HU control of the same strain, $^{*}P \leq 0.05$.

to exacerbate the severity of the CGG and CCG hairpin barriers. Since the absence of Srs2 did not show any significant effect on replication of stable protein–DNA complexes or G4-forming sequences, it appears that the Srs2 helicase specifically facilitates replication of hairpin-forming sequences. In the future, it will be important to test other hairpin-forming sequences of different lengths and base composition to further characterize the role of Srs2 in cells. Results with the Srs2 separation-of-function mutants suggest that the helicase activity, not the ability to displace Rad51, is needed for replication past the hairpin. In addition, the requirement for the PCNA-interaction domain suggests that Srs2 localization to the replication fork is a key factor in its ability to facilitate replication past this structural barrier. Srs2 was able to unwind both a CGG and CTG hairpin substrate *in vitro* at the same rate, supporting direct unwinding of the folded single strand as the mechanism used by Srs2 to facilitate fork progression past a hairpin. In contrast, the Sgs1, Pif1 and Rrm3 helicases did not facilitate replication of hairpin-forming CGG or CCG sequences, as measured by analysis of replication stalling. However, measurements of unwinding rates showed that Sgs1 was able to unwind a CGG hairpin *in vitro*, although less efficiently than Srs2. Based on our *in vitro* unwinding data, the difference between Srs2 and Sgs1 effects on CGGs can only partly be explained by their substrate specificity. It is likely that interaction with PCNA at the replication fork positions Srs2 in the right place at the right time as the fork encounters a hairpin structure. Previous data suggested that one role of PCNA-bound Srs2 is to control recombination at the replication fork (55–57). Our results now add the new

information that in WT cells, another role of Srs2 at the fork is to utilize its helicase activity to facilitate replication through DNA structures, probably by directed hairpin unwinding.

In addition to their hairpin forming capability, CGG repeats have been shown *in vitro* to be able to form G4 structures. It was not clear whether CGG/CCG repeats stalled replication due to their hairpin forming or G4 forming capability. Since both the strength and the nature of the CGG stalls were different than stalls induced by G_4T_4 and G_3T_2A and previously published 2D data with G_4T_2 sequences (26), a reasonable conclusion is that CGG repeats stall replication due to their hairpin formation rather than due to their G4 formation *in vivo*. Lack of an effect of deleting *Srs2* on all the G4 forming sequences compared to significant effect on CGG sequences is also consistent with this conclusion.

What could be the genetic consequences of increased fork stalling in the absence of Srs2? Our recombination assay, which indirectly measures the breakage rate of the triplet repeat, suggests that one of the consequences of increased fork stalling is increased chromosome breakage and recombination. The recombination rate was significantly higher when the CGG repeats formed the lagging strand template. This result is interesting given that the amount of stalled intermediates did not significantly differ between CGG and CCG repeats in the absence of Srs2. One explanation for the above result is that Srs2 also acts downstream of the stalled fork to prevent recombination between the duplicated LYS2 regions, either directly or by facilitating replication restart, and that there is a greater requirement for Srs2 activity in the CGG orientation where the hairpins are thermodynamically more stable than in the CCG orientation. Another explanation is that a higher state of negative supercoiling exists on the plasmid compared to the chromosome, allowing more frequent formation of CCG hairpins than in the chromosomal context. Our 2D analysis of replication of CGG repeats in the presence of HU showed no significant difference in the amount of stalled intermediates from the no-HU control, whereas previously it was shown that treatment with HU resulted in a moderate increase of CGG/CCG fragility (50). These results are consistent with the idea that fragility of CGG/CCG repeats is influenced by factors in addition to the degree of replication fork stalling.

G4-forming sequences have been suggested to stall replication forks and require specialized helicases to enable replication across these barriers (12). Our 2D data suggests that G4 DNA sequences do stall forks as we were able to detect replication pausing at the non-yeast telomeric sequences that was of a different nature than the yeast protein-bound repeat and was not affected by deletion of the Rrm3 protein. However, the G4-mediated stall was weaker in intensity than hairpin-forming sequences of equal G/C content (Figure 2e). G-rich sequences adopt quadruplex structures in neutral pH. Therefore one reason behind the weaker intensity of the stall due to G4-DNA could be the acidic environment inside yeast cells, disfavoring G4 formation. Indeed the C_4A_4 sequence, which can form an i-motif structure (favored under acidic conditions), was found to stall replication with approximately the same percentage as the G_4T_4 sequence. Alternatively, the stall in the C_4A_4 orientation could be due to G4 formation on the leading strand template or on the nascent Okazaki fragment, which would imply that G4 DNA can form in multiple locations with respect to a replication fork. Another reason for the weaker stall intensity could be the slow kinetics of G4 formation. Under standard *in vitro* conditions, G4 formation is observed after incubation of the G-rich strands ranging from minutes to hours (66). Thus a fast replicating fork, ~3000 bp/minute in eukaryotes (67), may preclude the formation of G4-DNA. Perhaps conditions where the G-rich strand is kept single-stranded for longer periods—for example by an active transcription bubble or at the end of telomeres—could allow for more frequent formation of G4 structures. In fact, G4 structures have been observed under such conditions (68,69).

Biochemical studies have shown that yeast Pif1, yeast Sgs1 and the Sgs1 human homolog BLM can efficiently unwind G4-DNA *in vitro* (15,16,58). If the Pif1 helicase does unwind G-quadruplexes, why did not we observe a role for Pif1 in unwinding the G_4T_4 and G_3T_2A sequences during replication? Interestingly, it was recently shown that there is a regional fork slowdown around some G4 motifs in *pif1* cells treated with HU, although notably this study also did not observe site-specific fork stalling in *pif1* cells, and identified maximal Pif1 binding after replication of the G4 motif (18). Paeschke *et al.* (18) suggest that Pif1 resolves G4 structures after S phase, and our results are consistent with this interpretation. Similarly, Sgs1 did not appear to have a direct role in facilitating fork progression through the G4 DNA. Perhaps these helicases act to unwind intermolecular G-quadruplexes formed between sister chromosomes after fork passage. Interestingly, *pif1Δ* cells showed increased stalling at a telomere sequence bound protein, even though this was not the case at the replication fork barrier (RFB) caused by Fob1p binding to rDNA (61). Pif1 has also been shown to release telomerase from telomeric oligonucleotides (70). Therefore Pif1 may have a special role in removing telomere-bound proteins.

We found that yeast telomeric sequences are formidable barriers to replication. Our results together with the results by Makovets *et al.* (26) provide strong evidence that these stalls are indeed protein mediated and not due to structure formation. First, the amount of pause signal induced by G_4T_4 and Htel sequences was significantly less than the Ytel stall even though they have similar G4 forming potential. Second, and consistent with its role as a protein sweepase, absence of Rrm3 exacerbated and altered the replication pattern of Ytel sequences. Third, absence of Tof1, which is required for the maintenance of a majority of pause sites at polar protein barriers, dramatically decreased the amount of the Ytel sequence pause signal.

Our analyses also revealed the Ytel sequences to be polar fork barriers that affected replication more when the G-rich sequence was replicated as the lagging strand template. In contrast, previous studies of fork stalling at an internal yeast telomeric sequence did not detect much

difference between the two orientations (26). This discrepancy could be due to several reasons: (i) the use of a more rigorous quantification analysis in our study, (ii) the location of the stall at the top of the Y arc in the previous study, where quantification is more difficult due to the variable degree of compression that naturally occurs at this location (see for example Figure 2a versus 2 d), or (iii) the use of a regular synthetic repeat in our study versus an irregular natural yeast telomeric sequence by Makovets *et al.* (26). Whatever the reason, we clearly see a 3–4-fold difference in the strength of fork stalling between the two orientations of the idealized yeast telomeric repeat, which is highly statistically significant. This difference was also observed in *srs2*Δ and *tof1*Δ strains, but was reduced to 2-fold in the *rrm3*Δ and *pif1*Δ mutants, suggesting a possible role for these helicases in establishing or maintaining the polarity (Figures 2 and 6, and data not shown). This polar fork barrier is likely due to the asymmetrical binding of a protein or protein complex that targets the Ytel sequence, such as mediated by Rap1 (25). What could be the biological meaning of the Ytel sequence acting as a polar fork barrier? Reduced stalling in the *tof1Δ* mutant suggests that similar to its role in the rDNA region, Tof1-mediated fork stalling at the Ytel sequences could promote recombination between Ytel sequences, providing a potential pathway for telomere homeostasis. For example, the processes described as telomeric rapid deletion (TRD) (71) and expansion of telomeric repeats by telomere–telomere recombination (72) could occur through a pathway of fork stalling-induced recombination. Evidence for stall-induced recombination can be seen in Ytel sequences replicated in *rrm3*Δ cells. Alternatively, slowing down replication could either facilitate error free replication or have a regulatory role.

The hydroxyurea experiments with CGG and Ytel sequences showed that depletion of dNTP pools did not affect the strength of the pausing at structure-forming sequences, whereas the amount of pausing was drastically reduced at the Ytel protein barrier. Moreover, the Ytel stall was completely abolished when replicated in the presence of HU and in the absence of Tof1. Previously it was suggested that HU, by means of inducing additional stalls in the genome, could titrate down Tof1 protein occupancy at regions of protein-mediated stalls. However, the additive effect of HU on the attenuation of the Ytel stall in *tof1Δ* cells suggests that slowing down replication acts independently of Tof1 to release this particular protein-mediated stall (23). It is known that in yeast, telomeres are replicated in late S phase when dNTP pools are likely to be diminished (67,73). Therefore our results with Ytel sequences suggest that under conditions of depleted dNTP pools, such as in late S phase, forks could traverse protein barriers more efficiently, and that late replication of yeast telomeric sequences in fact could be a way to replicate across these protein barriers.

In summary, we have identified distinct roles for different helicases in bypassing either protein or structural barriers in yeast cells, and shown that Srs2 helicase activity is particularly required for unwinding of stable hairpin structures to prevent replication fork stalling.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures 1–3.

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