Pif1 family helicases suppress genome instability at G-quadruplex motifs

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G-quadruplex (also known as G4) DNA is a four-stranded DNA structure held together by guanine (G) base pairing, and most genomes are replete with G4 motifs— that is, sequences that can form G4 structures in vitro. Several DNA helicases unwind G4 structures in vitro, including several human helicases (WRN, BLM, FANCJ and PIF1), the mutation of which is associated with genome instability, premature ageing and/or increased cancer risk (Supplementary Table 1).

In vitro

So far, more than 20 tested helicases, including both S. cerevisiae Pif1 and human PIF1, bind and/or unwind G4 structures in vitro (Supplementary Table 1). To determine whether S. cerevisiae Pif1 is particularly adept at unwinding G4 structures, we analysed its G4 binding and unwinding activities in a quantitative manner. Filter-binding assays were used to quantify Pif1 binding to different DNA substrates (Fig. 1 and Supplementary Fig. 1; oligonucleotides in Supplementary Table 2). Pif1 had a preference for poly-purine tracts (Fig. 1a), which was consistent with its preference for G-rich (dissociation constant \( K_d = 0.04 \) nM) over non-G-rich (\( K_d = 0.2 \) nM) single-stranded DNA (ssDNA) (Fig. 1c). Pif1 displayed similarly high binding to G4 DNA (average \( K_d = 0.08 \) nM for three G4 motifs; Fig. 1e), which was roughly 500-fold better than its binding to Y-structures (that is, in the presence of a 500-fold excess of unlabelled G4 DNA), it unwound G4 structures under single-cycle conditions with no change in kinetics (Fig. 2f). Thus, G4 structures are a preferred Pif1 substrate.

Pif1 is a potent G4 binder and unwinder

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Bacterial Pif1s are potent G4 unwinders

We are unable to purify full-length Rrm3, Pif1 (the Schizosaccharomyces pombe Pif1 family helicase), or human PIF1 (ref. 11). However, the sequences of many bacterial Pif1 proteins are available. To determine whether vigorous G4 unwinding is conserved among Pif1 family helicases, we purified Pif1 proteins from four diverse bacteria and a bacteriophage. All five enzymes robustly unwound the rDNA\(_{G4}\) and TP\(_{G4}\) substrates with apparent \( K_m \) values in the subnanomolar to nanomolar range (Fig. 2d and Supplementary Fig. 2–f). As with S. cerevisiae Pif1, each of these Pif1 family helicases unwound G4 DNA rapidly (Fig. 2e and Supplementary Fig. 2) and under single-cycle conditions (although not to completion) (Fig. 2c).

To determine whether a helicase is particularly good at G4 DNA unwinding, one can compare its unwinding of G4 DNA to the unwinding of other substrates (for example, S. cerevisiae Pif1 unwinding of G4 DNA versus Y-structures; Fig. 2a) or compare the unwinding activity of multiple helicases on the same G4 substrate. As several RecQ family helicases unwind G4 structures in vitro (Supplementary Table 1), we tested Sgs1, an S. cerevisiae RecQ helicase, and Escherichia coli RecQ. Sgs1 bound ssDNA and unwound Y-structures at reported rates (Fig. 2b). However, Sgs1 did not bind preferentially to G-rich DNA (Fig. 1b, d), and the apparent Sgs1 binding affinity for four G4

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structures was more than 40-fold lower than that of *S. cerevisiae* Pif1 (Fig. 1f). Similarly, Sgs1 was considerably less efficient than all tested Pif1 family helicases at unwinding G4 structures (for example, 1,000-fold molar excess of Sgs1 was needed to unwind 50% of the G4 structures; Fig. 2e). *E. coli* RecQ displayed better unwinding of the TP4 substrates than Sgs1 (Fig. 2c, e, f), but the apparent *K₅₀* value of this activity was 160-fold greater than that of Pif1.

Time-course experiments revealed slower unwinding of G4 structures by Sgs1 and *E. coli* RecQ (Fig. 2e) relative to Pif1, and Sgs1 was unable to unwind G4 DNA under single-cycle conditions. Although *E. coli* RecQ did unwind the TP4 substrate under single-cycle conditions (Fig. 2f), 500-fold more protein relative to Pif1 was necessary for activity, yielding a half-life (*t₁/₂*) approximately tenfold greater than that of Pif1 in the same assay. The same preparations of Sgs1 and *E. coli* RecQ unwind a conventional Y-structure 100- and 10-fold better, respectively, than G4 structures (Fig. 2b, c). With WRN, a human RecQ helicase, we obtained a similar unwinding rate for TPG4 and rDNA4, as reported for *E. coli* RecQ (ref. 17); both were similar to unwinding by Sgs1 (Fig. 2e and Supplementary Fig. 3). Thus, three evolutionarily diverse RecQ helicases were much less efficient than any tested Pif1 family enzyme at G4 unwinding.

**Pifs suppress G4-induced instability**

We developed a quantitative assay to monitor G4-induced genome instability by modifying the gross-chromosomal rearrangement (GCR) assay. The GCR assay detects complex genome rearrangements by simultaneous selection against *URA3* and *CANT* (Fig. 3a). We modified this assay by inserting four strong Pif1-binding sites, two G4 motifs (Chr IGR, Chr XGR) and two non-G4 sites (Chr VIII₄G₀ not G-rich; Chr IGR, G-rich, not G-forming; Supplementary Table 3), within the PRB1 locus, a non-essential gene that is centromere-proximal to the two counterselectable genes (Fig. 3a). As reported previously, the GCR rate in the ‘no insert’ wild-type control was approximately 1 × 10⁻¹⁰ events per generation, and none of the inserts affected this rate (Table 1). However, the already high GCR rate in the no-insert *pif1-m2* strain was increased ~3-fold in the presence of either of the G4 motifs but was unaffected by either of the other Pif1-binding sites (Table 1). The G4 inserts did not increase GCR rates in *rrm3Δ* or *sgs1Δ* cells compared to no-insert controls. Likewise, the GCR rate in *pif1-m2 sgs1Δ* cells was not increased by the G4 inserts. However, the GCR rate in *pif1-m2 rrm3Δ* cells was approximately eight times higher in the presence of the G4 motifs compared to the *pif1-m2 rrm3Δ* strains containing no insert or non-G4 Pif1-binding sites (1,700-fold over the background no-insert wild-type levels; Table 1). These data suggest that when Pif1 levels are low, Rrm3 (but not Sgs1) suppresses G4-induced genome instability. Consistent with these findings, Rrm3 bound preferentially to G4 motifs in *sgs1Δ* cells (Fig. 3b) and expressed from the *pif1-m2 rrm3Δ* strain was increased ~3-fold over the background wild-type level (Supplementary Fig. 3).

To determine whether Pif1 suppression of DNA damage at G4 motifs is evolutionarily conserved, we tested diverse Pif1 proteins for their ability to suppress the high GCR rate in *pif1-m2 rrm3Δ* G4 cells. Helicases were introduced on a single-copy plasmid and expressed from the *RRM3* promoter (see Supplementary Fig. 6 for western analysis of protein expression). A simple spot assay was used to monitor the frequency of GCR events; cells were spotted 150 times at high density on 5-fluoroorotic acid (5-FOA) plus canavanine sulfate (Can) plates, and incubated until resistant colonies formed (~20 GCR events per spot for the *pif1-m2 rrm3Δ* G4 strain containing no or empty vector; Fig. 4c). As expected, Pif1 and Rrm3 both suppressed the *pif1-m2 rrm3Δ* G4 GCR rate (0.06–0.09 events per spot), whereas helicase-dead *S. cerevisiae* Pif1 (Pif1-KA) did not
(19 events per spot; Fig. 4c). Remarkably, all seven heterologous Pif1 helicases, including human PIF1 (0.3 events per spot) and six prokaryotic/viral Pif1 helicases (0.07–1.0 events per spot), suppressed the high GCR rate.

**Novel G4-induced events in pif1 cells**

We used several methods to determine whether G4 motifs affected the structure of the distal portion of chromosome V in the GCR events (Figs 3 and 4a, b and Supplementary Figs 4c–e and 5). As predicted, multiplex PCR (Fig. 3b) and Southern blot (Supplementary Fig. 4c–e) analyses revealed that almost all GCR events in the no-insert pif1-m2 strain were due to telomere addition centromere-proximal to CAN1 (52 out of 56 events). However, apparent telomere addition was rare (5 out of 27) or not detected (0 out of 28) in GCR clones from pif1-m2 + G4 or pif1-m2 rrm3Δ + G4 cells, respectively (that is, CAN1 fragment retained in multiplex PCR (Fig. 3b)); new telomere bands were rare in Southern blots (Supplementary Fig. 4d, e)).

We also sequenced the 1,000-base-pair (bp) region around the G4 insert in individual GCR clones (Fig. 4a, b and Supplementary Fig. 5). There were no changes in this region in 17 out of 17 GCR clones from sgs1Δ + G4 cells. However, all (19 out of 19) G4 inserts were altered in pif1-m2 + G4 GCR clones. These changes included mutations limited to the G4 motif (5% of clones); partial or complete deletion of the G4 motif and/or flanking DNA (10%); and more complex events involving deletions, insertions, and inversions (85%) (Supplementary Fig. 5). A similar pattern was seen in most (82%) of the pif1-m2 rrm3Δ GCR events (Fig. 4 and Supplementary Fig. 5).

As expected, URA3 and CAN1 were lost or moved to new locations in all GCR clones from the wild-type strain containing a G4 insert (8 out of 8) and sgs1Δ + G4 (11 out of 11) cells. However, the positions of URA3 and CAN1 were unchanged in most pif1-m2 + G4 (19 out of 27) and pif1-m2 rrm3Δ + G4 (27 out of 28) GCR clones. On the basis of the high mutation rate of the G4 insert, we predicted that loss of URA3 and CAN1 expression would be due to mutations in the genes. However, cloning and sequencing of URA3 and CAN1 from six clones each from the pif1-m2 + G4 and pif1-m2 rrm3Δ + G4 strains revealed that the URA3 and CAN1 sequences, including a 200 bp up and downstream of the genes, were unchanged, but the left arm of chromosome V (Chr. V-L) was unstable in many of these clones. Subsequent analyses of the same clones, for example, after restreaks or growth in liquid culture, revealed that either URA3 or both URA3 and CAN1 were lost.

### Table 1 | Mean GCR rates

| Genotype | No insert | Chr lO4 | Chr X04 | G-rich | Non-G-rich |
|----------|-----------|---------|---------|--------|-----------|
| Wild type | 1.2 ± 0.5 | 1.4 ± 0.5 | 1.2 ± 0.2 | 1.3 ± 0.6 |
| pif1-m2 | 76 ± 8 | 200 ± 20 | 210 ± 10 | 70 ± 30 | 60 ± 20 |
| rrm3Δ | 6 ± 5 | 12 ± 8 | 9 ± 4 | 3 ± 1 | 3.2 ± 0.9 |
| sgs1Δ | 20 ± 80 | 190 ± 35 | 200 ± 50 | ND | ND |
| pif1-m2 rrm3Δ | 210 ± 32 | 1,500 ± 500 | 1,900 ± 200 | 200 ± 10 | 250 ± 40 |

Data are mean ± s.d. calculated from ≥3 independent experiments and normalized to the rate (1.5 ± 0.7) × 10⁻⁶ GCR events per generation in the wild-type strain with no insert at the PRB1 locus. ND, not determined.

Figure 3 | Effects of G4 motifs on GCRs. a, Schematic of Chr. V-L in GCR strains. Numbered bars denote the positions of PCR products in b. URA3 and CAN1 denote counterselectable markers; PCM1 is the most telomere-proximal essential gene. AlwNI sites are marked.

Figure 4 | Pif1 family helicases suppress G4-induced GCR events in pif1-m2 rrm3Δ + G4 cells. a, The G4-insert region was PCR-amplified and sequenced from 19 (pif1-m2 rrm3Δ) or 17 (others) GCR clones. b, Examples of G4 mutations. G-tracts in G4 formation are denoted by dashed boxes. Mutated G residues are in red; dashes denote deletions. c, GCR events in pif1-m2 rrm3Δ + G4 cells expressing the indicated helicase. Six out of one-hundred-and-fifty spots per strain are shown. GCR events are white colonies on a grey background of non-growing cells. The mean ± s.d. colonies per spot are indicated to the right. *P < 0.016 for the colonies per spot compared to the ‘no vector’ control as calculated by Student’s t-test. BdePif1, Bdellovibrio Pif1; BifPif1, Bifidobacterium Pif1; CamPif1, Campylobacter Pif1; hPIF1, human PIF1; PsyPif1, Psychrobacter Pif1; VPf1, V99B1Pif1.
as often as 95% of the time (data not shown). However, some clones maintained wild-type URA3 and CAN1 genes for ≥200 generations.

**Mechanism of G4-induced silencing**

Given that the sequences and positions of URA3 and CAN1 were unchanged in the pif1-m2+ G4, pif1-m2 rrm3A+ G4 and pif1-m2 rrm3A GCR clones that retained these genes, their 5-FOA- and Can-resistant (FOAR CanB) phenotypes must be due to epigenetic silencing. To determine whether the silencing occurred at the transcriptional level, we used real-time quantitative PCR (qPCR) to assess the amounts of URA3 and CAN1 messenger RNA in four independent pif1-m2 rrm3A+ G4 GCR clones and the parental pre-GCR strain. Depending on the clone, URA3 mRNA levels ranged from 9 to 24% of the levels in the pre-GCR strain; CAN1 mRNA ranged from 20 to 53% of the control in the same clones (Fig. 5a, b). Thus, silencing was not due to translational regulation.

In many organisms, including S. cerevisiae and humans,22,23 genes that are near telomeres are transcriptionally repressed (telomere position effect (TPE); reviewed in ref. 24). To determine whether the silencing observed in the pif1-m2+ G4 GCR clones was due to TPE, we deleted SIR2, which encodes a protein that is required for TPE, in two independent pif1-m2+ G4 GCR clones that retained URA3 and CAN1 in their original positions. Both clones lost their FOAR CanB phenotypes, suggesting that silencing was due to a TPE-like mechanism.

**Human PIF1 inhibits telomere lengthening**

Telomeres are longer in Pif1-deficient cells owing to the ability of S. cerevisiae Pif1 to remove telomerase from DNA ends2,3. To determine whether other Pif1 helicases inhibit yeast telomerase, we determined telomere length in pif1-m2 cells expressing heterologous Pif1 helicases (Fig. 5c). Empty vector or the expression of bacterial Pif1s (Bacteroides Pif1 and Campylobacter Pif1) or S. pombe Pif1 did not suppress the long telomere phenotype of pif1-m2 cells. Indeed, telomeres were even longer in pif1-m2 cells expressing bacterial Pif1s or Pfh1 than in pif1-m2 cells alone. However, human PIF1 was nearly as effective as S. cerevisiae Pif1 in restoring telomere length to pif1-m2 cells (Fig. 5c), even though it was expressed at much lower levels (Supplementary Fig. 6).

**Discussion**

S. cerevisiae Pif1 and five prokaryotic Pif1 helicases were extremely proficient at unwinding G4 structures (Fig. 2 and Supplementary Fig. 2), whereas three RecO helicases had ~1,000-fold lower G4 unwinding activity than the Pif1 helicases (Fig. 2 and Supplementary Figs 2 and 3). Moreover, although S. cerevisiae Pif1 unwound G4 structures much better than Y-structures (Fig. 2a), which are themselves a preferred Pif1 substrate13, Sgs1 and E. coli RecQ were more active on Y-structure than G4 structures (Fig. 2b, c). Thus, vigorous G4 unwinding is a conserved feature of Pif1 helicases.

Suppression of G4-induced DNA instability was also conserved (Table 1). In pif1-m2 cells, GCR rates were increased when the substrate contained a G4 motif but not when it contained other strong Pif1-binding sites; this effect is probably underestimated as pif1-m2 is not a null allele1. Similarly, the human minisatellite CEB1, a tandem array of ~40 [GC]-rich repeats, increases the GCR rate in pif1A cells23. In contrast to no-insert pif1-m2 cells, G4-mediated GCR events were rare due to telomere addition (52 telomere additions per 56 GCR events in pif1-m2 cells, versus 5 telomere additions per 27 GCR events in pif1-m2+ G4 cells). In both pif1-m2 and pif1-m2 rrm3A3 cells, the G4-induced events were usually associated with mutation of the G4 insert so that it could no longer form a G4 structure (Fig. 4a), suggesting that the process enabling cells to replicate and/or repair a G4 motif in the absence of Pif1 helicases is error-prone. Remarkably, the double drug-resistant phenotype of the G4-induced clones recovered from pif1-m2+ G4 (75 out of 104 clones) and pif1-m2 rrm3A+ G4 (50 out of 64 clones) was usually due to epigenetic silencing, although the genes could be lost during further outgrowth. Although silencing of URA3 and CAN1 in these complex genetic-epigenetic (CGE) clones was Sir2-dependent, as is TPE26, this silencing was considerably more effective than classical TPE. When URA3 is immediately adjacent to the chromosome VII-L telomere, mRNA levels are ~20% of control levels, but when URA3 is ~20 kilobases (kb) from the same telomere, FOAR colonies are not detected (~6 × 10⁻⁷). By contrast, in CGE clones, the average URA3 mRNA level was 19% of the control, even though URA3 was 21 kb from the telomere. The extension of silencing to more internal sites may be associated with impaired replication through a G4 structure, as changes in silencing occur in translesion polymerase-defective avian DT40 cells27. The unusual URA3 and CAN1 silencing seen here also required or was enhanced by lack of Pif1 and/or Rrm3, as it was not detected in gsg1A+ G4 GCR clones (0 out of 17 clones). Furthermore, it was enhanced by a nearby G4 motif as it was not seen in GCR clones from the no-insert pif1-m2 cells (0 out of 56 clones). The new events at both G4 motifs and structural genes in the absence of Pif1 family helicases are distinct from previously described GCR events. Thus, we term these CGE events. The epigenetic silencing of URA3 and CAN1 is reminiscent of the gene silencing that occurs in some human tumours that can lead to loss of heterozygosity.

Although Pif1 and Rrm3 have largely non-overlapping functions31, they both suppressed damage at G4 motifs, as did seven out of seven heterologous Pif1 helicases (Fig. 4c). This suppression was efficient. For example, human PIF1 suppressed CGE events ~20% as effectively as S. cerevisiae Pif1, even though it was expressed at considerably lower levels (Supplementary Fig. 6).

Figure 5 | Mechanism of CGE silencing and effect of Pif1 helicases on telomere length. a, URA3 (a) and CAN1 (b) mRNA levels in pif1-m2 rrm3A+ G4 CGE clones and controls (pre-GCR parental strains and ura3A and can1A cells). Quantitative reverse-transcriptase PCR (qRT–PCR) was used to determine the ACT1, URA3 or CAN1 mRNA levels in the indicated strains. URA3 and CAN1 values were normalized to ACT1 levels in each strain; the 2^(-ΔΔCt) method32 was used to determine URA3 and CAN1 levels relative to parental pre-GCR cells. *P < 0.05; **P < 0.01; ***P < 0.001. c, Telomere blot of DNA from pif1-m2 spore clones expressing vector only (lanes 1–3, 16, 17), ScPif1 (lanes 4–6, 18, 19), human PIF1 (7–9), two different bacterial Pif1s (10–12, 13–15), or Pif1 (20–22). M, markers. DNA was prepared ~50, 75 and 100 generations after sporation (first, second and third lanes in each set) or 100 generations after sporation from two or three spore clones (lanes 16–22). See Supplementary Fig. 7 for full gel images.
levels (Supplementary Fig. 6). Thus, activity at G4 DNA by both in vitro and in vivo assays is a conserved feature of Pif1 family helicases.

S. cerevisiae Pif1 (but not Rrm3 or Pfh1) inhibits telomerase4,29,29. Human PIF1 (but not prokaryotic Pif1 helicases or Pfh1) restored telomere length in pif1-m2 cells (Fig. 5c), suggesting that PIF1 is a regulator of both telomerase and G4 structures in its endogenous context. One or both of these activities might explain why mutation of human PIF1 is associated with cancer12.

METHODS SUMMARY
Strains were YPH500 (ref. 30) derivatives (Supplementary Tables 4 and 5). Cloning oligonucleotides are listed in Supplementary Table 6. The pif1-m2 allele was used instead of pif1A because pif1-m2 cells are mitochondrial proficient and grow at near-null-type rates. Pif1, Sgs1 and E. coli RecQ were purified and assayed as described14,15,16. Bacterial Pif1 helicases were cloned (Supplementary Table 8), overexpressed and purified as described in the Methods. G4 motifs were from the yeast genome10 or mouse immunoglobulin locus (TPG4) (Supplementary Information). G4 structures were formed in vitro12 and 5'-end labelled with [γ-32P]ATP. Protein–DNA binding was analysed by the double-filter binding method12. Activity of all Pif1 enzymes was measured as described previously12. WRN helicase assays were as described11. GCR assays were performed as described12. GCR rates were calculated using the FALCOR web server and MMS maximum likelihood method12. Multiplex PCR oligonucleotides are in Supplementary Table 7. G4 inserts were sequenced from genomic DNA and analysed with BioWorkbench tools (http://workbench.sdsc.edu/). Suppression analyses of GCR phenotypes were performed in pif1-m2 rrm3A+G4 cells carrying a single-copy TRP1-marked plasmid with 3'-Flag-tagged helicase genes under control of the RRM3 promoter (Supplementary Table 8). Chromatin immunoprecipitation was performed as described previously36. Total RNA was isolated using a Quick-RNA MiniPrep kit (Zymo Research), reverse-translated into complementary DNA using an iScript One-Step RT–PCR kit with SYBR Green (Bio-Rad), and analysed by real-time PCR using a Bio-Rad CFX96 real-time system.

Full Methods and all associated references are available in the online version of the paper.

Received 6 August 2012; accepted 5 April 2013.

Published online 8 May 2013.

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METHODS

Methods Summary. Strains were YPH500 (ref. 30) derivatives (Supplementary Tables 4 and 5). E. coli RecQ was purified and assayed as described. G4 motifs were from the yeast genome or mouse immunoglobulin locus (TPR-α) (Supplementary Tables 2 and 3). G4 structures were formed in vitro and 5' end labelled with [γ-32P]ATP. Protein–DNA binding was analysed by the double-filter binding method. WRN helicase assays were as described. GCR assays were performed as described. GCR rates were calculated using the FALCOR web server and MMS maximum likelihood method.

Yeast strains. All experiments were performed in the YPH500 background. Yeast strains are listed in Supplementary Table 4, except for those used in the GCR assays (Supplementary Table 5). Gene disruption and epitope tagging of proteins were confirmed by colony PCR, sequencing, Southern blotting and/or phenotypic analysis. The pif1-m2 allele was introduced as previously described (see Supplementary Table 6 for oligonucleotide sequences used for cloning). The carboxy terminus of Rrm3 was tagged at its endogenous locus with 13 Myc epitopes using PCR. Tagged Rrm3 was expressed from its own promoter as the only version of the protein in the strain. Plasmids are listed in Supplementary Table 8. All GCR strains (Supplementary Table 5) are derivatives of YPH500 in which HXT13 was deleted with the Klyuyeromyces lactis URA3 gene using pUG72 (ref. 40) and oligonucleotides MB262 and MB277 (see Supplementary Table 6 for the sequences of oligonucleotides used in GCR strain construction). The partial loss of nuclear function pfj1-m2 allele was used instead of pfj1Δ because pfj1-m2 cells are mitochondrial proficient. RRM3 was deleted with HIS3MX6 using pFA6α-His3MX6 (ref. 41) and oligonucleotides MB30 and MB31. SG1 was deleted with the S. pombe his5Δ genome using pUG27 (ref. 40) and oligonucleotides MB32 and MB33 (Supplementary Table 6). Strains containing ‘inserts’ (Supplementary Table 5) were made by deleting PBR1 with LEU2 marked cassettes using oligonucleotides KP321f and KP321r (Supplementary Table 8). The LEU2 marked cassettes were derived from prS415-based plasmids containing the designated inserts cloned into the XbaI and BamHI sites (Supplementary Table 8).

Biochemical methods. Full-length S. cerevisiae Pif1 and Sg1 and E. coli RecQ were purified as previously described. In vitro analyses of independent protein preparations revealed little to no prep-to-prep variability and that these preparations had similar biochemical activities (that is, ssDNA binding and Y-structure DNA unwinding, see below) to previously published variants.

Bacterial Pif1 helicases were cloned as follows. E. Allen-Vercoe, C. Parker, R. Johnson and H. L. Ayala-del-Rio provided genomic DNA from Bacteroides sp. 2_1_16, Campylobacter jejuni subsp. jejuni NCTC 11168, E. coli phage rv5 and Psychrobacter sp. PBWf-1, respectively. A pUC19-based plasmid containing the gene encoding the Bdelovirio bacteriovorax HD100 Pif1 helicase (Supplementary Table 8) was a gift from E. Sackett. PCR primers were designed to amplify the Pif1-like helicase genes from the above mentioned organisms (see Supplementary Table 6) with or without an N-terminal His6 tag (BioRad). PCR products were then digested and ligated into a modified pET21d vector (pMB116; Supplementary Table 8) such that they were in-frame with an amino-terminal 4× Strep-tag II sequence and a C-terminal 6× His tag. Additional cloning details and nucleotide sequences are available on request.

Expression plasmids were transformed into Rosetta 2(DE3) pLyS8 cells and selected for at 37°C on Luria–Bertani (LB) medium supplemented with 100 μg ml−1 ampicillin and 34 μg ml−1 chloramphenicol. Fresh transformants were used to inoculate one or more 5-ml LB cultures supplemented with antibiotics and incubated at 30°C for ~6 h with agitation. These starter cultures were then diluted 1:100 in ZYP-5052 auto-induction medium containing 1× trace metals mix mg2+, 100 μg ml−1 ampicillin and 34 μg ml−1 chloramphenicol, and incubated at 30°C with agitation to an OD (at 600 nm) of >3 (~18 h). Cells were collected by centrifugation for 10 min in a GS-3 rotor at 4,225g and 4°C. Cell pellets were weighed and frozen at −80°C before lysoz for long-term storage.

The cells were thawed at room temperature and resuspended in 2 ml N2-1× cell pellet buffer (50 mM Na-HEPES, pH 8, 10% (v/v) glycerol, 500 mM NaCl, 5 mM MgCl2 and 0.05% (v/v) HECAMEG) using 30-kilodalton (kDa) or 50-kDa filter binding method. WRN helicase assays were as described. GCR assays were cloned and performed essentially as described.

For some protein preparations, the N-terminal 4×Strep II tag was removed by PreScission Protease (GE Healthcare) digestion (2 U protease per ml protein at 4°C overnight) before His60 column chromatography. In all cases, removal of the tag had little effect on subsequent protein purity and no effect on the in vitro activities examined. However, tag cleavage occasionally resulted in precipitation of a considerable portion of the protein. Thus, recombinant proteins containing both N- and C-terminal tags were used for all experiments shown.

For preparation of substrates, various S. cerevisiae G4 motifs were chosen from the >500 identified G4 motifs in the budding yeast genome (see Supplementary Tables 2 and 3 for sequences). Oligonucleotides of G4 motifs were synthesized by IDT. The concentrations of all oligonucleotides were estimated using extinction coefficients provided by the manufacturer. G4 DNA structures were formed in vitro as described. Formation of G4 structures was confirmed by non-denaturing PAGE. After G4 structure formation, the substrates were 5'-labelled with T4 polynucleotide kinase (NEB) and [γ-32P]ATP, purified by 7% non-denaturing PAGE, and visualized using phosphoimaging.

In all biochemical assays, 100 pM radioactively labelled DNA was used, unless noted otherwise, and the reaction buffers used were previously described for S. cerevisiae (refs. 14, 41). In brief, protein–DNA binding was analysed using a BioDot SF apparatus (Bio-Rad) by the double-filter binding method. Reactions were set up as for helicase assays, but ATP was omitted. The reactions were incubated on ice for 30 min, filtered through the membranes, and then the membranes were washed with additional reaction buffer. The membranes were dried and analysed by phosphoimaging. Pif1, Sg1 and RecQ helicase activity assays were performed as previously described. For protein titrations, reactions were incubated for 30 min at helicase at 25°C (50 pM Pif1, 30°C (50 pM Sg1), or 37°C (RecQ, WRN, and non-yeast Pif1). In time-course experiments, 100 pM ScPif1, 10 nM BacPif1, 10 nM Sg1 or 50 nM EcRecQ was added to the reaction; 100 pM Sg1 or EcRecQ displayed only basal levels of unwinding in our G4 unwinding assays. For single-cycle conditions, we used a 500× excess of either G4 DNA or ssDNA as a protein trap. The excess trap DNA was added together with ATP to start the reactions.

The data were fit with rectangular hyperbolic curves using GraphPad Prism 5 and equation (1):

\[ Y = \frac{Y_{\text{max}} \cdot X}{K + X} \] (1)

in which X is the helicase concentration or time (as indicated), Y is either DNA binding or unwinding (as indicated), Y_{\text{max}} is the maximum level of binding or unwinding (as indicated), and K is the midpoint of the curve. When a log_{10}-scale x axis is used, the hyperbolic curve assumes a sigmoidal shape.

GCR assays. GCR assays were cloned and performed essentially as described (primer sequences for cloning are listed in Supplementary Table 8). In brief, sets of five or more 5-ml cultures of each S. cerevisiae GCR strain (Supplementary Table 5) were grown to saturation in YPD medium at 30°C for 36–48 h. A final dilution of 1× 10^{-7} of each culture was plated on YPD and incubated at room temperature for 4 days to determine the viable cell count. Cells (1.5 or 2 ml) from each culture were pelleted, resuspended in sterile water, plated on dot-dilution medium used in GCR assays (Supplementary Table 8) supplemented with 1 g l^{-1} 5-FOA and 60 mg l^{-1} canavanine sulphate (FOA+Can), and incubated at 30°C for ~4 days. GCR rates were calculated using the FALCOR web server and MMS maximum likelihood method and normalized to wild-type rate of 10^{-10} GCR events per cell division. The rates presented in Table 1 are the mean ± s.d. of
mids containing C-terminally 3
by transforming strain KP326 (Supplementary Table 5) with
mutated to other residues or deleted; and (4) mutation/deletion/insertion, the
workbench.sdsc.edu/) and classified into four different categories, as indicated in
Supplementary Table 8). Three independent
results and multiplex PCR, such bands are not likely to be telomere
additions.

Suppression analyses of the pif1-m2 rm3A + G4 GCR phenotype were performed by
transforming strain KP326 (Supplementary Table 5) with TRPI-marked plasmids containing C-terminally 3× Flag-tagged helicase genes expressed under control of the RRM3 promoter (Supplementary Table 8). Three independent colonies were used to inoculate 5 ml synthetic complete medium lacking trypto-
phan (SC–Trp) and grown on a roller drum for ~48 h at 30 °C. The D600nm for each culture was determined with a spectrophotometer, and the cells were pelleted by centrifugation and resuspended to D600nm = 10 in sterile H2O. Then, a repeat pipetter was used to spot 10-μl samples of each strain 50 times on a FOA + Can plate, and the plates were incubated at 30 °C for 4 days. This process was repeated ≥3 times for each strain. When colonies appeared on the FOA + Can plates, the number of colonies per 10-μl spot was counted, and the average number of col-
onies in the 50 spots per plate was calculated. The mean (± s.d.) of these values from the ≥3 plates per strain was determined and reported in the right column of Fig. 4c.

Western and Southern blotting. Cell extracts for western blotting were prepared as described previously44. In brief, cells were grown overnight in SC–Trp liquid medium at 30 °C with aeration. Then, 1 ml of D600nm ≥ 2.5 cells was collected, resuspended in 200 μl 0.1 N NaOH, incubated at room temperature for 5 min, pelleted, resuspended in 50 μl SDS–PAGE sample buffer, boiled for 3 min, and pelleted again. Subsequently, 6 μl of the supernatants was loaded onto an 8%
l SDS–PAGE sample buffer, boiled for 3 min, and 40 cycles of
C for 10 min, 95
C (for primer sequences, see Supplementary Table 7). The proteins were transferred to a nitrocellulose membrane at 4
melting. The oligonucleotides used for sequen-
cing results and multiplex PCR, such bands are not likely to be telomere
additions.

Chromatin immunoprecipitation. Chromatin immunoprecipitation (ChIP) of asynchronous yeast cells growing in rich medium was performed as described25,26 and analysed using an iCyclerIQ Real-Time PCR detection system (Bio-Rad Laboratories). Rrm3 was C-terminally tagged with 13 Myc epitopes26. An anti-
Myc monoclonal antibody (Clontech 631206) was used as the anti-serum in ChIP. The amount of DNA in the immunoprecipitate was normalized to the amount in input samples. The ChIP experiment was analysed by qPCR in duplicate or triplicate to obtain an average value for each sample. The ChIP experiment was repeated ≥3 times at each locus. For each qPCR experiment, the amount of signal in the Rrm3 immunoprecipitated was normalized to input and to the immu-
noprecipitated signal from ARO1, a sequence that contains no candidate G4 DNA motif and that has low Rrm3 association26.

Multiplex PCR. In brief, genomic DNA isolated from S. cerevisiae strains before and after GCR events was analysed by multiplex PCR using the primer pairs in Supplementary Table 7 and the following cycling parameters: initial denaturation for 5 min at 95 °C, 35 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 45 s, and a final extension at 72 °C for 10 min. PCR products (10 μl per reaction) were run at 90 V on 2.5% agarose gels containing ethidium bromide and visualized by ultraviolet transillumination (for primer sequences, see Supplementary Table 7). qPCR. The indicated strains were grown in FOA + Can liquid media for 12 h and then transferred to YEPD for 12 h until reaching a concentration of D600nm = 0.5, and total RNA was isolated using a Quick-RNA MiniPrep kit, including the DNase I treatment, as described by the manufacturer (Zymo Research). cDNA was synthesized from 200 ng DNase I-treated RNA using an iScript One-Step RT–PCR kit with SYBR Green (Bio-Rad) and analysed qPCR using a Bio-Rad CFX96 real-time system. The following primers were used: URA3 cDNA, 5′-GGTCCGACTGTAGCTTACGAATC-3′ and 5′-CGCAGATCCCTCTATACTGAAATC-3′; CAN1 cDNA 5′-AATAATCACTCGGCGGTTCAC-3′ and 5′-TCAGAAAGCITCAATATCC-3′; ACT1 cDNA, 5′-GTAACATGTTAGTCGGTGTAATC-3′ and 5′-CCAAATGACCACTCACCACAG-3′. The cycling parameters were: 50 °C for 10 min, 95 °C for 5 min, and 40 cycles of
95 °C for 10 s followed by 57 °C (ACT1), 50 °C (CAN1), or 55 °C (URA3) for 30 s. The data were analysed by the 2-ΔΔCt method39.

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