

# Pif1 regulates telomere length by preferentially removing telomerase from long telomere ends

Jing-Ru Li<sup>1</sup>, Tai-Yuan Yu<sup>2</sup>, I-Chieh Chien<sup>2</sup>, Chia-Ying Lu<sup>3</sup>, Jing-Jer Lin<sup>2,3,\*</sup> and Hung-Wen Li<sup>1,\*</sup>

<sup>1</sup>Department of Chemistry, National Taiwan University, Taiwan, <sup>2</sup>Institute of Biopharmaceutical Sciences, National Yang-Ming University, Taiwan and <sup>3</sup>Institute of Biochemistry and Molecular Biology, National Taiwan University, Taiwan

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## ABSTRACT

**Telomerase, a ribonucleoprotein complex, is responsible for maintaining the telomere length at chromosome ends. Using its RNA component as a template, telomerase uses its reverse transcriptase activity to extend the 3'-end single-stranded, repetitive telomeric DNA sequence. Pif1, a 5'-to-3' helicase, has been suggested to regulate telomerase activity. We used single-molecule experiments to directly show that Pif1 helicase regulates telomerase activity by removing telomerase from telomere ends, allowing the cycling of the telomerase for additional extension processes. This telomerase removal efficiency increases at longer ssDNA gaps and at higher Pif1 concentrations. The enhanced telomerase removal efficiency by Pif1 at the longer single-stranded telomeric DNA suggests a way of how Pif1 regulates telomerase activity and maintains telomere length.**

## INTRODUCTION

Telomerase replicates the repetitive DNA sequence at chromosome ends in eukaryotic cells (1). The repetitive DNA sequence, telomere, protects cells from the end replication problem and abnormal DNA degradation (2). Telomerase is responsible for maintaining the telomere length. The elevated activity of telomerases in proliferation cells, such as cancer cells and stem cells (3), makes the telomerase a potential therapeutic target in cancers and aging-associated disease (4,5).

Pif1 proteins have been shown to regulate telomerase activity (6). Pif1, a 5'-to-3' helicase, belongs to the super family I (SF1) helicase and is a prototype of Pif1 family conserved from yeast to humans (7,8). Most studied members of Pif1 family were reported to have the capability of maintaining telomere stability (9) and of unwinding G-quadruplex (10,11). *Saccharomyces cerevisiae* Pif1 is found in both mitochondria and nucleus. The mitochondrial Pif1 participates

in DNA repair, recombination, and maintaining mitochondrial genome (12–14), whereas the nuclear Pif1 is indispensable for break-induced replication (BIR) repair and stimulates DNA synthesis during BIR and crossover recombination (15,16). Moreover, Pif1 prevents genome instability at G-quadruplex forming sequences (17,18). Pif1 helicases had been reported to associate with telomerase activity both in *S. cerevisiae* (6,19,20) and humans (9,21). Both genetic and biochemical evidences suggest that Pif1 displaces telomerase from telomere to regulate telomere extension. This is consistent with the preferential unwinding activity of Pif1 on the RNA/DNA hybrid substrate which exists in the telomerase–telomere stalled complex (22). Mutations in the telomerase finger domain resulted in a recovery of the negative regulation imposed on telomerase by Pif1 (23), indicating also the existence of the protein–protein interaction in the Pif1–telomerase complex.

The detailed mechanism of how Pif1 regulates telomerase activity remains unclear. Specifically, how Pif1 directly displaces telomerase from telomere ends remains uncharacterized. Here we used several single-molecule experiments to directly monitor the regulation of Pif1 on telomerase activity. An *in vitro* telomere extension assay has been developed to facilitate the detection of multiple rounds of telomere extension. Our results showed that the telomerase–telomere stalled complex is stable but can be disrupted by Pif1 helicase. We also demonstrated that the efficiency of this disruption depends on Pif1 concentrations. Significantly, we found telomerase removal efficiency increases at long ssDNA gap substrates. Taken together, our results suggest a model that multiple Pif1 can effectively remove telomerase from long telomere ends, which in turn modulates the telomerase activity and the telomere length.

## MATERIALS AND METHODS

### Purification of yeast telomerase

Plasmid pRS426-GAL-EST2-TAP carrying TAP-tagged Est2 was transformed into yeast strain CY123 harboring

\*To whom correspondence should be addressed. Tel: +8862 3366 4089; Fax: +886 2 2363 6359; Email: hwli@ntu.edu.tw  
Correspondence may also be addressed to Jing-Jer Lin. Tel: +886 2 2312 3456; Fax: +886 2 23915295; Email: jingjerlin@ntu.edu.tw

plasmid pRS424-GAL-TLC1 (from Dr. Jin-Qiu Zhou, Chinese Academy of Sciences, China) (24). The resulting yeast cells were grown in medium containing 2% raffinose at 30°C until OD<sub>600</sub> to 1.0. Galactose (3%) was then added to the cells to induce telomerase expression and continued to culture for another 12–16 h. The TAP-tagged telomerase was purified following protocol described by Puig *et al.* (25). The eluted proteins were divided into aliquots and frozen directly using a dry ice/ethanol bath.

### Purification of yeast Pif1

The *Escherichia coli* expression system for recombinant yeast Pif1 was kindly provided by Dr. V. A. Zakian (Princeton University, USA). The polyhistidine-tagged Pif1 was expressed and purified from *E. coli* Rosetta strain (Novagen) using the bacterial expression pET28b (Novagen) system that was described by Boulé *et al.* (20). Briefly, cells were grown in Luria-Bertani (LB) media at 37°C until OD<sub>600</sub> ~0.6. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the culture to a final concentration of 1 mM and then incubated overnight at 23°C. Induced cultures were harvested by centrifugation, resuspended in buffer A (50 mM Sodium Phosphate pH 7.0, 300 mM NaCl) and lysed by sonication. The lysate was cleared by centrifugation and the supernatant was incubated with Talon resin charged with Co<sup>2+</sup> (BD Biosciences). The bound proteins were eluted by washing the column with Buffer A containing 200 mM imidazole. Fractions containing Pif1 were pooled and dialyzed against Buffer B (50 mM NaOAc pH 4.6, 200 mM NaCl, 50 mM Mg(OAc)<sub>2</sub>, 50 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>). The resulting proteins were then mixed with Bio-Rex 70 resin and the bound proteins were eluted with a 200 mM to 1 M NaCl gradient. Fractions containing pure Pif1 were pooled, concentrated, fast frozen in dry ice/ethanol bath and stored at -80°C in buffer C (25 mM HEPES pH 8.0, 100 mM NaCl, 25 mM Mg(OAc)<sub>2</sub>, 50 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1 mM DTT, 50% glycerol).

### Gel electrophoresis unwinding assay

For helicase assays, 1 mM top strand oligonucleotide (5'-CGCCATGGTGATCCGAGTGC-3' for the DNA substrate) was <sup>32</sup>P-labelled with T4 polynucleotide kinase, mixed with equimolar amounts unlabelled bottom strand oligonucleotide (5'-CACTGGCCGTCTTACGGTCGGC-ACTCGGATCACCATGGCG-3' for the DNA substrate), heated to 95°C, cooled overnight to 4°C and gel purified. 1 nM gel purified substrate was incubated in the presence of the indicated amount of purified Pif1 in buffer containing 20 mM Tris-HCl at pH 7.5, 50 mM NaCl, 5 mM MgCl<sub>2</sub>, 100 mg/ml bovine serum albumin (BSA), 10 mM adenosine triphosphate (ATP) and 2 mM DTT with an ATP regeneration system (50 mM creatine phosphate and 50 mg/ml creatine phosphokinase). Products were analyzed on 12% non-denaturing gels and quantified on a PhosphorImager (Molecular Dynamics).

### DNA preparation

All DNA substrates containing ssDNA gaps used in single-molecule experiments were first prepared by polymerase

chain reaction (PCR) reactions, followed by the restriction digestion of nicking endonuclease Nt.BstNBI (New England Biolabs, NEB). The nicked DNA substrates were heated to 98°C and annealed with excess DNA oligos (p45) to form dsDNA substrates containing 20 nt 5'-ssDNA overhang. The annealed DNA substrates were then purified and annealed with DNA oligos of different lengths of poly dT nucleotides with the telomeric sequence at the 3' end (Integrated DNA technology), and ligated using T4 DNA ligase (NEB). Sequences of DNA substrates are shown in supplemental tables.

### Streptavidin and calmodulin-coated beads

Streptavidin- and calmodulin-coated beads were prepared by covalently coupling the 220 nm carboxylate polystyrene beads (Bangs Lab.) with streptavidin (Prozyme) or calmodulin (Sigma) in carboimide hydrochloride (Merck) and sulfo-N-hydroxysulfosuccinimide (Fluka). Excess glycine (Merck) solution (1 M) was used to quench the coupling, and the free streptavidin or calmodulin was removed by repeating the spin-and-wash steps 10 times using phosphate buffer.

### Telomerase calmodulin-bead coupling

Purified telomerase was incubated at 0.8 nM with calmodulin-coated beads at 1x telomerase extension buffer (10 mM Tris-HCl pH 8.0, 2 mM Mg(OAc)<sub>2</sub>, 1 mM spermidine, 1 mM DTT) for 2 h at 4°C, followed by a spin and wash step using 1x Pif1 reaction buffer.

### Tethered particle motion (TPM)-based *in vitro* telomerase activity assay

For the reactions without additional specifications, the bead-labeled telomerases (up to 0.8 nM) were mixed with DNA substrates (4 nM) containing telomeric sequence in the presence or absence of Pif1 helicase at 30°C with specified deoxyribonucleotide incubated for overnight. For experiments carried out at different incubation times, the reactions were carried out at 7 nM of DNA substrates and telomerase concentrations up to 2 nM, depending on batches of telomerase purification used. For the higher DNA to enzyme ratio experiments, telomerase concentration was ~1 nM and the DNA concentration was 80 nM. The extension reactions were stopped by introducing RNaseH and Protease K (NEB) at 30°C for 20 min before heated at 80°C to disrupt telomerase-DNA interaction. The whole mixture was subjected to centrifugation to remove bead-labeled telomerases. The DNA substrates were found in the supernatant and immobilized onto the surface as previous described. To visualize the extent of telomerase-mediated telomere lengthening, we incubated immobilized substrates DNA with 5' biotinylated oligo probe (CC-CACACACACC, 5 μM) for 20 min. Streptavidin-coated polystyrene bead were introduced into the reaction chamber for tether visualization. Due to the sequence of oligo probe are complementary to the front part of telomere sequence (#1, Supplementary Table SI (A)), the oligo probe could be annealed to several different positions in the case of multiple rounds of telomere extension.

### Slide preparation for single-molecule assays

We prepared slides for single-molecule imaging as described previously (26). The anti-digoxigenin (20 ng/ $\mu$ L) was incubated firstly, followed by BSA blocking at 1mg/ml. DNA substrates solution (1 nM) was introduced for surface immobilization. The 1x Pif1 reaction buffer (20 mM HEPES pH 8.0, 50 mM NaCl, 5 mM MgCl<sub>2</sub>, 2 mM DTT) was used for single-molecule imaging experiments.

### Image acquisition and analysis

Single molecule tethering events were observed under an inverted microscope (Olympus IX-71) with differential interference contrast image method. The imaging acquisition was at 30 Hz. The Brownian motion amplitude (BM) was defined as the standard deviation of the centroid position of the tethers with an average of 40 frames, and the value of BM were drift-corrected using a stuck bead pre-adsorbed on the surface.

## RESULTS

### Pif1 enhances multiple rounds of telomerase-mediated telomere extension

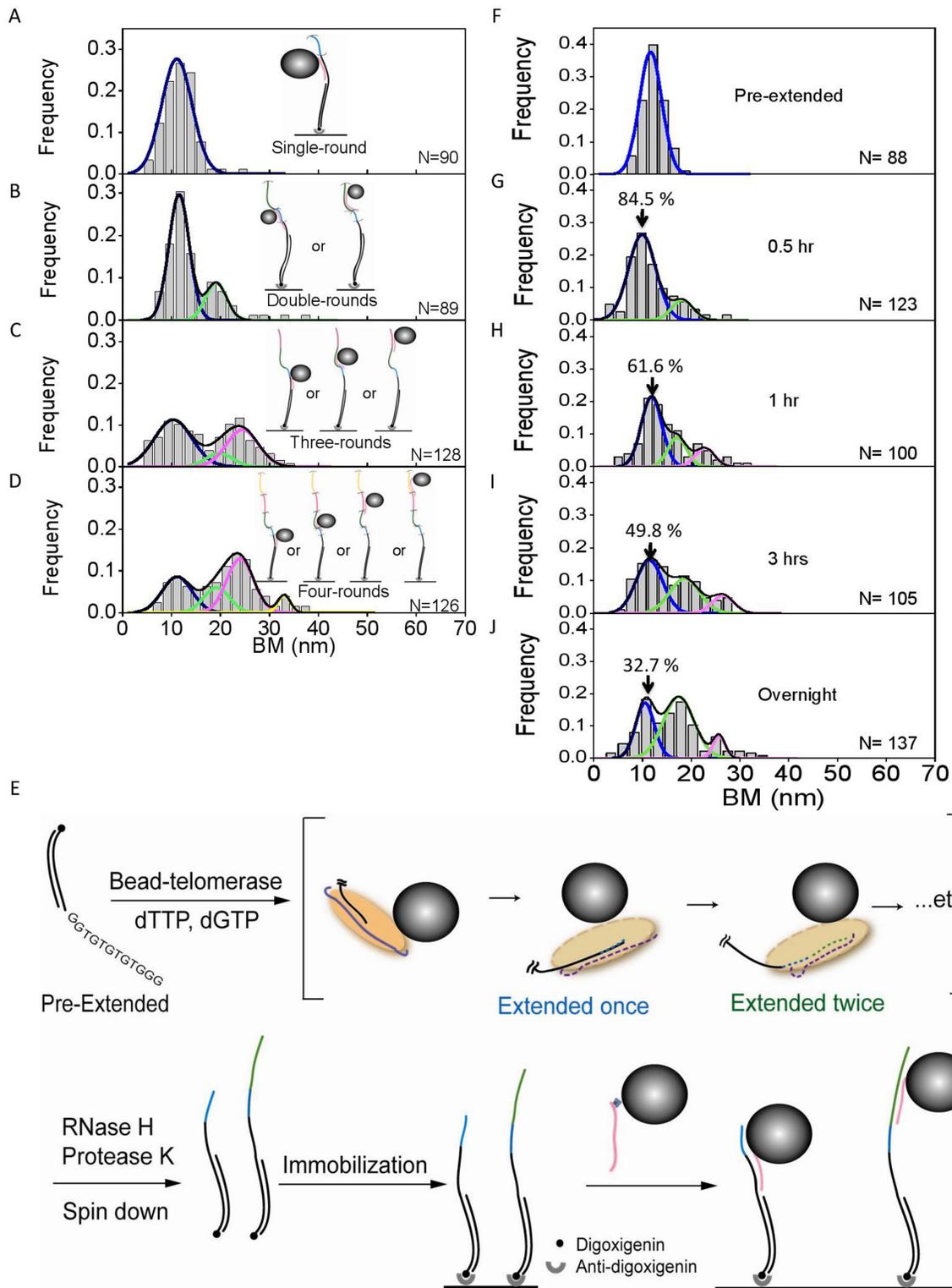
Telomerase ribonucleoprotein complex uses its RNA template to anneal to the ssDNA telomere end and extend the repetitive sequence for the telomere length maintenance. It had been suggested that multiple catalytic cycles of telomerase must take place in coupled with either complex translocation or complex dissociation/re-association events (27). Conventional primer extension assay monitors the products of the telomerase-mediated telomere extension based on gel electrophoresis (28,29). However, due to the sensitivity of the gel assays and the amount of DNA required, the assay is limited to observe mostly single-round or double-rounds DNA extension activity by yeast telomerases. PCR amplification of the primer-extended products can also be used for monitoring telomerase activity *in vitro* (30), but the assay cannot differentiate whether the extended products are single-round or multiple-rounds extension. To investigate the regulation mechanism of telomerase action, we developed a single-molecule telomere extension assay based on the TPM experiment to monitor the multiple-rounds of telomerase-mediated telomere extension process.

We first tested whether the observed BM amplitudes of the TPM extension assay could reflect the telomerase-mediated telomere extension. Here we prepared various 5'-digoxigenin labeled DNA substrates of different lengths that mimicked the ssDNA extension products after one, two, three and four rounds of extensions by telomerase (see Supplementary Table SI (D) for DNA substrates). The DNA substrates were immobilized onto the anti-digoxigenin-coated slide surface, and were then annealed to oligo probes coated by 220 nm polystyrene beads. These individually tethered DNA-beads undergo BM, which can be visualized under an optical microscope, and BM distributions were then analyzed. The mimicked 'single-round' extension DNA substrate showed a single BM peak at 11.1  $\pm$  3.0 nm (mean  $\pm$  SD, Figure 1A). In the case of a multiple-rounds extension, the probe oligos can anneal to several dif-

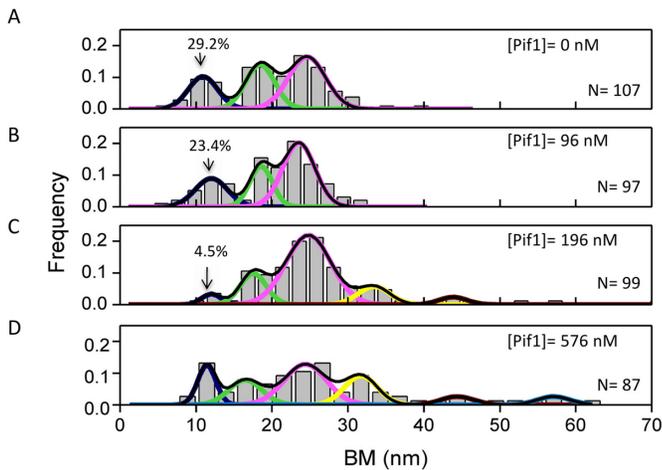
ferent positions, due to the repetitive nature of the telomere sequences. As expected, DNA substrates that mimicked 'double-rounds' of extensions showed two peaks, with an additional, higher BM peak at  $\sim$ 19.1 nm (Figure 1B). Similarly, DNA substrates with 'three-rounds' extensions showed peaks at  $\sim$ 10.6, 18.0 and 23.9 nm, and those with 'four-rounds' extensions showed peaks at  $\sim$ 11.1, 17.2, 24.0 and 33.3 nm (Figure 1C and D). Significantly, we found a linear relationship between the number of extended rounds and the observed BM peak values (Supplementary Figure S1A). Thus, our TPM-based assay is capable of detecting multiple-rounds of telomerase-mediated telomere extension products.

In our experiments to monitor-multiple rounds of telomerase-mediated telomere extension (Figure 1E), the 5'-digoxigenin labeled DNA substrates (#I) containing telomere sequences were first incubated with yeast telomerase for telomere extension. At different duration of reaction time, the mixture was treated with protease K and RNaseH to remove the telomerase. The extended DNA were then purified and immobilized onto the anti-digoxigenin-coated slide surface, followed by the annealing of the same oligo probes coated by 220 nm polystyrene beads for TPM analysis. The DNA substrates alone showed BM readout around 12.1  $\pm$  2.3 nm (Figure 1F). One larger BM peak (18.3 nm) showed up when the DNA substrates were incubated with telomerase in 30 min (Figure 1G). The percentage of larger BM peaks gradually appeared and increased and that of the lowest BM peak (representing the non-extended and single-rounds substrates) decreased at the longer reaction times, showing that the telomeric DNA substrates were extended by telomerases (Figure 1H–J, Supplementary Figure S1B). These larger BM values are consistent with those observed in the mimicked substrates (Figure 1A–D). As a control experiment, addition of dATP and dCTP did not extend the DNA substrate at overnight (11.3  $\pm$  3.9 nm, Supplementary Figure S1C). These experiments demonstrated the feasibility of the developed TPM-based telomerase extension assay in monitoring the multiple-rounds extension activity of telomerases. Due to the repetitive telomere sequences, the population of each BM peak only offers the lower limit in terms of how many rounds of the extension have been carried out. When the reaction was carried out at a higher DNA to telomerase ratio, the BM peak remained in 12.4 nm with no any additional BM peak seen after 1 h incubation (Supplementary Figure S1D), suggesting that there was no multiple-round extension under this condition.

We then investigated how Pif1 regulates telomerase extension activity. Figure 2 showed that at higher Pif1 concentrations both the DNA population at high BM peaks (extension more than single-round) and the largest BM peak value (maximum extension round) increase. We estimated that only 4.5% of DNA tethers remained in the pre-extended state at 196 nM of Pif1 (Figure 2C), indicating that >95% of telomeric DNA tethers was extended at least twice by telomerases, a large increase from that the reactions without Pif1 (29.2% of the lowest BM peak, shown in Figure 2A). Higher BM peaks at  $\sim$ 33.4 and 43.8 nm were also apparent at high Pif1 concentration. The 33.4 nm peak was consistent with the BM values of four-rounds of telomere extension (Figure 1D). Increased population(s) of higher



**Figure 1.** Observation of the multiple-rounds telomerase-mediated telomere extension. (A–D) BM histograms of the control custom-prepared DNA substrates mimicking the telomerase-extended products. (A) BM histogram of the mimicked ‘single-round’ DNA substrates. (B) Mimicked ‘double-rounds’ BM histogram. Since the oligo probe can anneal to two repetitive sequences in the ‘double-rounds’ DNA substrate, there are two BM peaks with the higher BM peak representing the ‘double-rounds’ population. (C) Mimicked ‘three-rounds’ BM histogram. (D) Mimicked ‘four-rounds’ BM histogram. (E) TPM method to monitor the *in vitro* multiple rounds telomerase extension activity. DNA substrates with same telomere sequence in the 3'-end were incubated at 30°C with bead-labeled telomerase and specified nucleotides. The telomerase-mediated telomere extension reactions were stopped by protease K and RNase H. The purified DNA substrates were then immobilized in glass surface and annealed with oligo probes, followed by TPM analysis. (F) BM histogram of DNA without telomerase incubation. (G–J) BM histogram of DNA substrates incubated with telomerase in the presence of dTTP and dGTP for 0.5 h, 1 h, 3 h and overnight. The percentage of the lowest BM peak (non-extended and extended once) decreases with the reaction time.

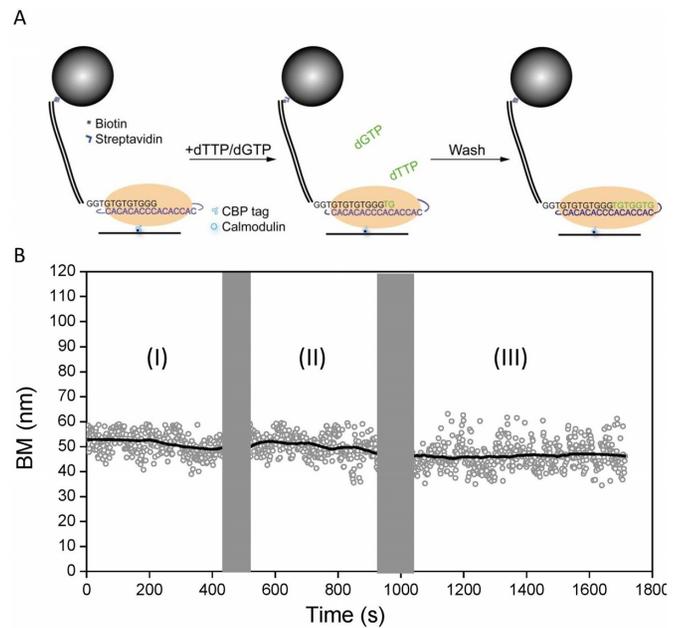


**Figure 2.** Pif1-stimulated multiple rounds telomerase extension activity depends on Pif1 concentrations. (A–D) BM histograms of the telomere extension at different Pif1 concentrations from 0 to 576 nM. Higher BM-value peaks reflect the longer extended telomere DNA products.

BM peaks as well as more peaks in higher BM demonstrated directly that ATP-dependent Pif1 helicase activity stimulates the telomerase-mediated multiple rounds telomere extension. This result agrees with the previous report of increased single-round extension products in the presence of Pif1 helicase (20). This observation of Pif1 stimulation on the telomerase-mediated telomere extension is also seen at shorter time scale (Supplementary Figure S1E). At the highest Pif1 concentration used, an apparent BM peak at  $\sim 45$  nm and a few more data points at even higher BM  $\sim 55$  nm were observed, suggesting that at least five-rounds of telomerase-mediated extensions can be detected in this Pif1 concentration. The population of the pre-extended BM peak ( $\sim 11$  nm) was slightly increased at this high Pif1 concentration, probably caused by the competition of substrate binding between telomerases and high concentration of Pif1. Earlier work in yeast and human both suggested that Pif1 could regulate the telomerase extension activity by removing telomerase from telomere ends using its helicase activity (6,19,20,22). The Pif1 concentration-dependent stimulation of the telomerase extension activity observed here might be resulted from either multiple Pif1 molecules interact together in the extended ssDNA telomere complex and remove telomerase efficiently, or multiple Pif1 increase the overall turning-over probability of telomerases. This kind of the Pif1 regulation model requires that the dissociation of telomerase from telomere ends to be the rate-limited step, so we next examined the stability of the telomerase–telomere complex.

### Telomerase–telomere complex is stable

We directly monitored the stability of the telomerase–telomere complex at the single-molecule level (Figure 3A). *S. cerevisiae* telomerase with a calmodulin binding peptide (CBP) tag was immobilized on the calmodulin-coated glass surface. A 537 bp DNA substrate (#S) containing a 220 nm streptavidin-labeled bead attached to the 5'-end with telomere sequence at its 3'-end overhang was in-



**Figure 3.** Telomerase–telomere complex is stable. (A) The calmodulin-labeled and surface-immobilized yeast telomerase binds to the telomere-containing and bead-labeled DNA. The telomerase contains a RNA template with sequence complementary to the end of telomere-containing DNA substrates (537 bp dsDNA with 12 nt ssDNA overhang). Deoxyribonucleotides dTTP and dGTP were introduced to initiate the extension reaction. After 5 min, the extension complex was washed using reaction buffer without dTTP and dGTP. (B) The exemplary time course showed that the telomerase–telomere tether was stably tethered, with the BM amplitude unchanged and consistent with the DNA handle designed ( $51.8 \pm 2.5$  nm, Supplementary Figure S2). Region I represents the time before telomere extension. Stalled complex stayed bound even after the addition of 50  $\mu$ M deoxyribonucleotides (dTTP and dGTP) for at least 5 min (Region II). It also remained tethered after extensive buffer wash to remove free nucleotides (Region III). On average,  $>84\%$  of tethers stayed bound over the entire time courses.

roduced into the reaction chamber and captured by the surface-bound telomerase. The stability of the telomerase–telomere complex was then monitored by the presence of DNA tethers. We found that the pre-extension telomerase–telomere complex was very stable that the bead remained bound to the surface for more than 400 s (Figure 3B, Region I). Addition of dTTP/dGTP to initiate the telomere extension did not appear to affect the stability of the telomerase–telomere complex (Region II). The post-extension telomerase–telomere complex remained stable for more than 10 min even after the depletion of deoxyribonucleotides using extensive buffer wash (Region III). The observed BM of the complex are consistent with the expected DNA substrate length ( $51.8 \pm 5.0$  nm, Supplementary Figure S2) and did not show apparent BM change within the experimental resolution during  $\sim 30$  min time course of tethering. On average,  $>84\%$  of the observed telomerase–telomere complex stayed tethered over the entire time courses, confirming the stability of the complex. The results indicate that the telomerase remains stably bound to the telomere before and likely after one cycle of extension, consistent with the previous observation (28,31). Since the telomerase–telomere complex is long-lived, dis-

sociation and recycling of telomerases from telomere ends serve as potential targets for regulating the telomerase activity.

### Pif1 helicase requires >5 nt ssDNA gaps for efficient unwinding

Biochemical analysis suggested that Pif1 could use its helicase activity to disrupt the RNA/DNA hybrid in the telomerase–telomere complex, thus facilitating the dissociation of telomerases (20,22). To test the efficiency of the Pif1 unwinding activity, we used the single molecule TPM method to study the ssDNA gap dependence in unwinding 15 bp duplex DNA/DNA substrates. Digoxigenin-labeled DNA substrates (#HT1-#HT4) containing 537 bp duplex DNA and various lengths of poly dT ssDNA overhangs between the telomere sequence and duplex DNA were first immobilized on the slide surface through anti-digoxigenin antibody. The biotin-labeled 15 nt oligonucleotides with the sequence complementary to the end of the telomere sequence were coupled with streptavidin-coated beads and annealed to form 15 bp long unwinding DNA-bead tethers (Figure 4A). Pif1 helicases is thought to load onto the ssDNA gap and initiate the unwinding the 15 bp long DNA/DNA in a 5'-to-3' direction. The disappearance of the tethered bead is a consequence of the helicase unwinding, and is used as a signal of the completed unwinding. Our TPM-based Pif1 unwinding analysis showed that a ssDNA gap is required for Pif1 to unwind and to remove tethered beads since 0 nt gap returns no apparent unwinding (Figure 4B). The Pif1 unwinding efficiency, defined as the percentage of tether disappearance, increases and reaches its maximum when the ssDNA gap is 5 nt or longer.

A parallel conventional gel electrophoresis-based unwinding assay was also used to confirm the helicase activity (Figure 4C). In the gel assay, substrates DNA (#G1-G4) carrying various gapped ssDNA between a stem loop and a tailed, isotope-labeled 16 bp dsDNA. Unwinding products were analyzed by gel electrophoresis. Same as what we observed in the single-molecule TPM method, the gel-based assays also showed that the Pif1 helicase requires a ssDNA gap with the unwinding efficiency reaching a maximum when the ssDNA gap is larger than 5 nt long (Figure 4D). Therefore, as long as ssDNA gap is 5 nt or longer, Pif1 helicase can efficiently load onto the DNA substrate, and unwind at least 15 bp long DNA/DNA with high efficiency.

### Direct observation of telomerase removal by Pif1 helicase

We next developed a single-molecule assay to directly observe the telomerase removal from telomere ends by Pif1 helicase (Figure 5A). Similarly as in Figure 4, the DNA substrates (#HT5-#HT8) were attached to the surface through the digoxigenin/anti-digoxigenin linkage. Telomerase was labeled with calmodulin-tagged beads through its CBP tag. DNA tether formation took place when the RNA template of telomerase anneals to the complementary ssDNA sequence at the 3'-end of the surface-anchored DNA substrates (Figure 5A). Successful telomerase removal by Pif1 helicase can then be observed by the disappearance of beads. Using this telomerase removal assay, we found Pif1

removed telomerase in an ATP-dependent manner when the ssDNA gap is 58 nt long (Figure 5B, solid bar). Nearly 50% of the observed beads were removed by Pif1 within 30 min. The telomerase-removing activity by Pif1 helicase required ATP hydrolysis since a non-hydrolyzable ATP analogue, ATP $\gamma$ S or ATPase mutant Pif1<sup>K264A</sup> did not show successful removal events (Figure 5B), consistent with previous reports that ATP hydrolysis is required for Pif1 helicase activity (6,20,32,33). Our TPM results provide a direct evidence for the removal of telomerase from telomeric DNA ends by Pif1 helicases.

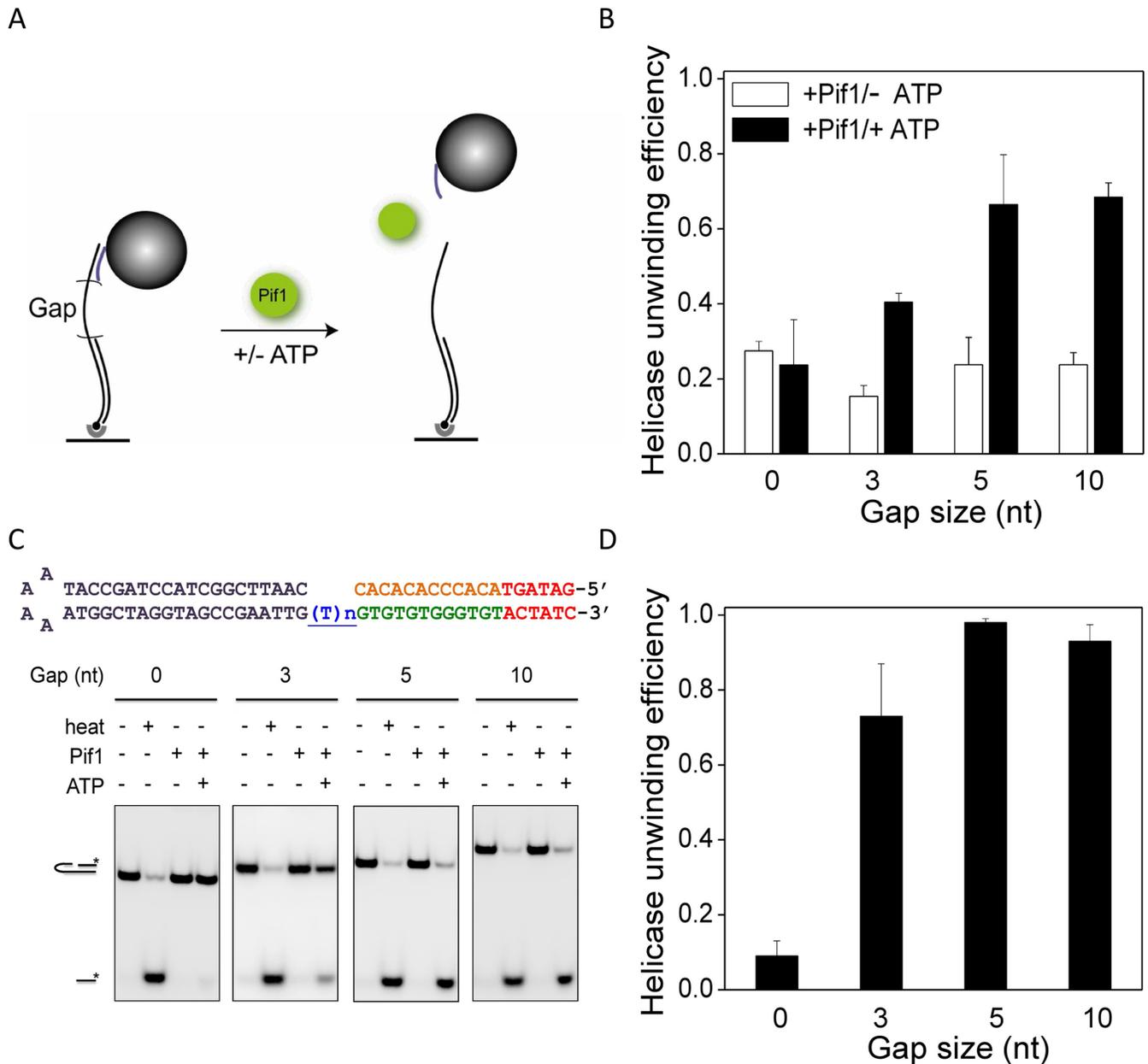
### Pif1 efficiently removes telomerase from DNA with a long ssDNA gap

Even though the telomerase removal is apparent at 58 nt ssDNA gap, no apparent removal was detected when the ssDNA gap is three nucleotide long (Figure 5C). We then tested a series of DNA substrates carrying ssDNA gap size of 34, 46, 58 and 82 nts for both telomerase removal (Figure 5C) and Pif1 unwinding (Figure 5D) processes at 25°C and 30°C, respectively. Surprisingly, the longer the ssDNA gaps, the greater the Pif1-stimulated telomerase removal efficiency (Figure 5C). The removal efficiency was increased from ~0.3 to ~0.5 when the ssDNA gap was increased from 34 to 82 nucleotides. The gap-size-dependent telomerase removal by Pif1 was also observed when the experiments were performed at a higher temperature (30°C, Supplementary Figure S3). In strong contrast, Pif1 unwinding activity was all very efficient for these long ssDNA gap substrates (all about the maximum efficiency seen in Figure 4B, Figure 5D). This is not surprising since Pif1 helicase is capable of unwinding DNA/DNA as long as the substrates contain ssDNA gap longer than 5 nt (Figure 4B).

The durations of the telomerase–telomere complex during Pif1 removal process among these DNA substrates have also been studied. As show in Supplementary Figure S4, the durations of the telomerase–telomere complex decreased from ~1400 s to less than 500 s when the ssDNA gap increased from 34 to 82 nucleotides. The duration of the telomerase–telomere complex reflects the kinetic step of the Pif1 removal process. The positive correlation is observed for the shorter duration and the higher telomerase removal efficiency at longer ssDNA gaps (Supplementary Table SII). Thus, Pif1 efficiently removes telomerase from DNA with a long ssDNA gap with an enhanced rate.

## DISCUSSION

Our experiments demonstrated a few important direct observations that help to elucidate the functional mechanism of how Pif1 helicase regulates the telomerase-mediated telomere extension process *in vitro*: First, the telomerase–telomere stalled complex is thermodynamically stable; second, Pif1 helicase stimulates the multiple telomere extension cycles by telomerases under the limited DNA substrate condition; and third, Pif1 helicase removes telomerase from telomere ends, and the efficiency of this process is enhanced at longer ssDNA gaps. Our helicase unwinding experiments also showed that Pif1 is capable of effectively unwinding 15 bp DNA/DNA duplex as long as a ssDNA gap larger than

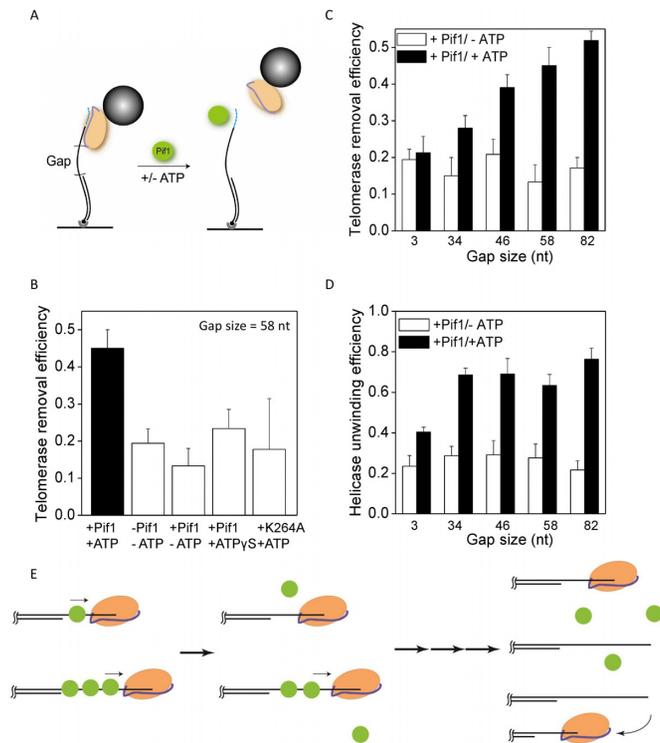


**Figure 4.** Pif1 helicase requires ~5 nt ssDNA gap for efficiently unwinding dsDNA substrates. (A) The single-molecule, single-turnover Pif1 helicase unwinding assay. DNA substrates containing telomere sequence at the 3'-end overhang were immobilized on the slide surface and were annealed to bead-labeled, oligonucleotide probes complementary to the telomere sequence to form DNA tethers containing a 15 bp DNA/DNA duplex at the end. Tethers were then challenged by Pif1 helicase (24 nM) in the presence of 10 mM ATP. Successful Pif1-catalyzed unwinding events were reported by the disappearance of beads. (B) The unwinding efficiency of Pif1 helicase on 15 bp dsDNA at various ssDNA gap sizes in the presence of 10 mM ATP (solid bar) and without ATP (empty bar) at 30 min at 30°C. Each data points include more than three independent experiments with more than 50 DNA tethers in each experiment. (C) The gel electrophoresis unwinding assay using stem-loop DNA substrates containing 5'-end P<sup>32</sup>-labeled strand (\*) with various ssDNA gap sizes. Unwinding condition was exactly same as in (B). (D) Quantification results of the gel image shown in (C).

5 nt. The >5 nt ssDNA gap size observed for effective unwinding is consistent with the predicted sit size of Pif1 on ssDNA (34), and likely reflects the minimal ssDNA site for Pif1 loading. We also showed that Pif1 is even more efficient in unwinding the canonical telomerase–telomere complex (RNA/DNA duplex) given the required minimal ssDNA site (>5 nt). Helicase unwinding experiments using RNA/DNA substrates indeed return with better efficiency at 5 min reaction time, with no gap size dependence seen

at larger ssDNA gaps, consistent with our DNA/DNA observation (Supplementary Figure S5). However, the telomerase removal efficiency by Pif1 increases in the ssDNA gap ranging from 34 to 82 nt. It is unlikely that Pif1 removes telomerase purely based on its helicase unwinding activity to disrupt interaction between RNA template and telomere DNA.

Increasing evidence suggests that many helicases function as ssDNA translocases (35,36). By translocating along



**Figure 5.** Efficiency of the Pif1-stimulated telomerase removal from the telomere complex depends on the ssDNA gap size, but the Pif1 unwinding has the same efficiency over this gap size range. (A) The single-molecule telomerase removal assay by Pif1. DNA substrates containing the telomere sequence at the 3'-end were immobilized on the slide surface. Bead-labeled telomerase recognized the telomere sequence and formed stable tethers in the presence of dTTP and dGTP. The telomerase-DNA complexes were then challenged by Pif1 helicase in the presence of 10 mM ATP. Successful telomerase removal events were identified by the disappearance of beads. (B) Telomerase-telomere complex disruption requires Pif1 helicase and ATP hydrolysis. The significant telomerase removal process was only observed in the presence of wild-type Pif1 helicase, and ATP hydrolysis. Experiments without ATP in the presence and absence of Pif1, or with 10 mM non-hydrolyzable ATP analogue, ATPγS, or with the Pif1 ATPase deficient mutant K264A (empty bars) showed only basal level of telomerase removal efficiency. Data includes more than three independent experiments at 30 min at 25°C, with more than 30 DNA tethers in individual experiments. (C) Telomerase removal efficiency is higher at longer ssDNA gaps in the presence of 10 mM ATP (solid bars) at 30 min reaction time at 25°C. This telomerase removal by Pif1 requires ATP hydrolysis, since reactions in the absence of ATP (empty bars) returned with the same background disappearance level. It is noted that with three nucleotides gap size, there is no significant difference in the presence or absence of ATP conditions, showing that single-strand DNA binding is necessary to telomerase removal. (D) The Pif1 helicase unwinding efficiency measured by TPM (Figure 4A) for 34–82 nt ssDNA gaps is all very high in the presence of 10 mM ATP at 30 min (solid bars). (E) Models for how Pif1 helicase regulates the telomerase activity. The long telomere provides more ssDNA loading sites for multiple Pif1 at higher Pif1 concentration, with the enhanced telomerase removal efficiency and telomere lengthening.

single-stranded DNA, helicase exerts force on the DNA substrates. This ssDNA translocase activity not only unwinds duplex DNA but also, more importantly, removes any encountered proteins bound to the DNA as it translocates (37), so to make the DNA available as substrates for downstream biochemical processes. Even more, there are examples of helicases to effectively remove roadblocks (such as protein-bound DNA) using an oligomeric state, as seen

in Dda helicase and Rep helicase (38,39). In the example of the Dda helicase, the Dda monomer–monomer work cooperatively to remove streptavidin effectively at the end of DNA substrates (40). A recent study showed the ssDNA length dependence in the efficiency of Pif1 displacing streptavidin from the biotin-labeled ssDNA, and suggested that multiple Pif1 molecules aligning on the ssDNA and exhibiting modest cooperativity in the obstacle displacement (32). Since we observed the ssDNA gap size dependence of Pif1-stimulated telomerase removal (Figure 5C) as well as the Pif1-concentration dependence on the telomere extension (Figure 2) at given ssDNA gap size, we investigated the possibility of multiple Pif1 helicases in removing telomerases.

Compared to the high efficiency in unwinding the RNA/DNA or DNA/DNA duplex substrate, the efficiency of Pif1 helicase to successfully remove huge ribonucleoprotein telomerase complex from DNA is rather low. The simplest model for Pif1 helicases to efficiently remove telomerase from telomere ends is that multiple Pif1 bind to the ssDNA gap but work independently. Single Pif1 helicase is unlikely to remove telomerase in a single encounter with high efficiency, but the efficiency of overall successful removal can be increased with multiple Pif1 helicases in line. In this simple Pif1 encounter model, we assume first, the site size of Pif1 helicase is ~6–8 nt ssDNA, second, at the Pif1 concentration used, the ssDNA gap is fully occupied by Pif1 helicases and third, each Pif1 would encounter telomerase once and dissociate even not successful in removing it. It was previously reported that Pif1 do not remove streptavidin roadblock cooperatively even multiple Pif1 could align on the long ssDNA substrates (32,33). Based on these assumptions and statistics arguments, the effective removal efficiency observed experimentally (defined as the difference in the removal efficiency between the experiments and control from Figure 5C) is, surprisingly, consistent with the simple, independent Pif1 encounter model proposed (Supplementary Figure S6). Within our experimental resolution, the probability for single Pif1 to successfully remove telomerase during a single encounter is found to be very low, ~0.04 (1-q, with q as the failed probability, Supplementary Figure S6). However, when several Pif1 helicases encounter telomerase consecutively, the probability of successful removal increases. Small or modest cooperativity among Pif1 helicases could exist, but independently multiple and consecutive Pif1-telomerase encounter events play a major role in the successful removal process seen in our long ssDNA gap substrates. Even though this concept was implicated in the prior Pif1 displacing streptavidin experiments, the report here demonstrates how Pif1 removes telomerase in maintaining the telomere length.

Our results suggested that the telomerase activity might be up-regulated by Pif1 helicase, with the level of regulation dependent on the ssDNA telomeric gap size and Pif1 concentration. When the concentration of Pif1 is low or the ssDNA telomere is short, the monomer Pif1 helicase has low removal efficiency, resulted in the telomerase-bound telomere and short telomere. On the contrary, high concentration of Pif1 or long telomere ssDNA gap provides higher probability of Pif1 loading, leading to the enhanced telomerase removal efficiency (Figure 5E), which in turn allows the recycling of telomerase to access other telomere ends. Since

extensive *in vivo* and biochemical analyses have suggested that Pif1 might function as a negative regulator to modulate telomerase activity (6). The *in vitro* stimulation observed here implies that there must exist additional pathways interacting with Pif1 for telomerase modulation directly or indirectly. Defining these molecular interaction pathways *in vitro* is essential for regulating the telomerase activity.

Our finding for the preferential removing of telomerase from long ssDNA by Pif1 could have several implications. First, the ssDNA gap size dependence of telomerase removal by Pif1 helicases seen here could potentially support the previous finding that Pif1 deletion recovered the temperature sensitivity and the increased overall telomere length in the yeast *ku* mutant cells (41). Yeast *ku* mutants have been shown to have long telomere overhangs and short telomeres. Increased resection at telomeres in *ykuΔ* strains causes long telomere overhangs that could potentially allow Pif1 to efficiently remove telomerases. Therefore, Pif1 may load on the long telomere overhang and remove telomerase in *yku* mutants to prevent the extension of telomeres. Second, our data suggest that Pif1 functions as a regulatory factor to remove telomerase from long ssDNA telomere ends. Considering the Pif1 level are regulated by cell cycles, this telomerase removal from long ssDNA telomere ends can allow Pol $\alpha$  and Pol $\delta$  to effectively conduct lagging strand synthesis (42,43). This removal might allow telomerase to access other shorter telomeres. Third, our results are consistent with an early *in vivo* studies that the rate of *de novo* telomere formation after HO-induced chromosome breakage was elevated in *pif1* mutants (19). During double-stranded DNA breaks repair, the DNA ends undergo extensive resection to generate long ssDNA. Pif1 might remove telomerase from these ssDNA tails to prevent erroneous telomere addition by telomerase. Preferential removal of telomerase from longer ssDNA by Pif1 may help to prevent the harmful telomere addition at double-strand breaks.

Put together, we directly observed that Pif1 removes telomerase from the telomere–telomerase stalled complex at the single-molecule level. At the longer ssDNA gap, multiple Pif1 helicases effectively remove telomerase from telomere ends, allowing the cycling of telomerase for additional extension processes. This telomerase removal process by Pif1 helicases offers a way to regulate telomerase activity at the long telomere ends. The single-molecule extension assay developed here furnishes a useful means to investigate the regulation of the telomere maintenance by other telomere-binding proteins. Future experiments employing other telomerase regulatory components will help to characterize the interaction among these regulatory proteins as well as how telomeres are partitioned among different molecular pathways.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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## REFERENCE

- Greider, C.W. and Blackburn, E.H. Greider, C.W. and Blackburn, E.H. (1985) Identification of a specific telomere terminal transferase activity in Tetrahymena extracts. *Cell*, **43**, 405–413.
- Levy, M.Z., Allsopp, R.C., Futcher, A.B., Greider, C.W., and Harley, C.B. Levy, M.Z., Allsopp, R.C., Futcher, A.B., Greider, C.W., and Harley, C.B. (1992) Telomere end-replication problem and cell aging. *J. Mol. Biol.*, **225**, 951–960.
- Counter, C.M., Avilion, A.A., LeFeuvre, C.E., Stewart, N.G., Greider, C.W., Harley, C.B., and Bacchetti, S. Counter, C.M., Avilion, A.A., LeFeuvre, C.E., Stewart, N.G., Greider, C.W., Harley, C.B., and Bacchetti, S. (1992) Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. *EMBO J.*, **11**, 1921–1929.
- Flores, I., Canela, A., Vera, E., Tejera, A., Cotsarelis, G., and Blasco, M.A. Flores, I., Canela, A., Vera, E., Tejera, A., Cotsarelis, G., and Blasco, M.A. (2008) The longest telomeres: a general signature of adult stem cell compartments. *Genes Dev.*, **22**, 654–667.
- Tomás-Loba, A., Flores, I., Fernández-Marcos, P.J., Cayuela, M.L., Maraver, A., Tejera, A., Borrás, C., Matheu, A., Klatt, P., and Flores, J.M. Tomás-Loba, A., Flores, I., Fernández-Marcos, P.J., Cayuela, M.L., Maraver, A., Tejera, A., Borrás, C., Matheu, A., Klatt, P., and Flores, J.M. (2008) Telomerase reverse transcriptase delays aging in cancer-resistant mice. *Cell*, **135**, 609–622.
- Zhou, J.Q., Monson, E., Teng, S.-C., Schulz, V.P., and Zakian, V.A. Zhou, J.Q., Monson, E., Teng, S.-C., Schulz, V.P., and Zakian, V.A. (2000) Pif1p helicase, a catalytic inhibitor of telomerase in yeast. *Science*, **289**, 771–774.
- Bessler, J.B., Torredagger, J.Z., and Zakian, V.A. Bessler, J.B., Torredagger, J.Z., and Zakian, V.A. (2001) The Pif1p subfamily of helicases: region-specific DNA helicases?. *Trends cell biol.*, **11**, 60–65.
- Bochman, M.L., Sabouri, N., and Zakian, V.A. Bochman, M.L., Sabouri, N., and Zakian, V.A. (2010) Unwinding the functions of the Pif1 family helicases. *DNA Repair*, **9**, 237–249.
- Zhang, D.-H., Zhou, B., Huang, Y., Xu, L.-X., and Zhou, J.-Q. Zhang, D.-H., Zhou, B., Huang, Y., Xu, L.-X., and Zhou, J.-Q. (2006) The human Pif1 helicase, a potential Escherichia coli RecD homologue, inhibits telomerase activity. *Nucleic Acids Res.*, **34**, 1393–1404.
- Sanders, C.M. Sanders, C.M. (2010) Human Pif1 helicase is a G-quadruplex DNA-binding protein with G-quadruplex DNA-unwinding activity. *Biochem. J.*, **430**, 119–128.
- Paeschke, K., Capra, J.A., and Zakian, V.A. Paeschke, K., Capra, J.A., and Zakian, V.A. (2011) DNA replication through G-quadruplex motifs is promoted by the Saccharomyces cerevisiae Pif1 DNA helicase. *Cell*, **145**, 678–691.
- Foury, F. and Kolodnynski, J. Foury, F. and Kolodnynski, J. (1983) pif mutation blocks recombination between mitochondrial rho+ and rho- genomes having tandemly arrayed repeat units in Saccharomyces cerevisiae. *Proc. Natl. Acad. Sci. U.S.A.*, **80**, 5345–5349.
- Lahaye, A., Stahl, H., Thines-Sempoux, D., and Foury, F. Lahaye, A., Stahl, H., Thines-Sempoux, D., and Foury, F. (1991) PIF1: a DNA helicase in yeast mitochondria. *EMBO J.*, **10**, 997–1007.
- Lahaye, A. and Leterme, S. Lahaye, A. and Leterme, S. (1993) PIF 1 DNA helicase from Saccharomyces cerevisiae. *J. Biol. Chem.*, **268**, 26155–26161.
- Saini, N., Ramakrishnan, S., Elango, R., Ayyar, S., Zhang, Y., Deem, A., Ira, G., Haber, J.E., Lobachev, K.S., and Malkova, A. Saini, N., Ramakrishnan, S., Elango, R., Ayyar, S., Zhang, Y., Deem, A., Ira, G., Haber, J.E., Lobachev, K.S., and Malkova, A. (2013) Migrating bubble during break-induced replication drives conservative DNA synthesis. *Nature*, **502**, 389–392.
- Wilson, M.a., Kwon, Y., Xu, Y., Chung, W.-H., Chi, P., Niu, H., Mayle, R., Chen, X., Malkova, A., and Sung, P. Wilson, M.a., Kwon, Y., Xu, Y., Chung, W.-H., Chi, P., Niu, H., Mayle, R., Chen, X., Malkova, A., and Sung, P. *et al.* Wilson, M.a.,

- Kwon, Y., Xu, Y., Chung, W.-H., Chi, P., Niu, H., Mayle, R., Chen, X., Malkova, A., and Sung, P. (2013) Pif1 helicase and Pol $\delta$  promote recombination-coupled DNA synthesis via bubble migration. *Nature*, **502**, 393–396.
17. Myung, K., Chen, C., and Kolodner, R.D. Myung, K., Chen, C., and Kolodner, R.D. (2001) Multiple pathways cooperate in the suppression of genome instability in *Saccharomyces cerevisiae*. *Nature*, **411**, 1073–1076.
18. Paeschke, K., Bochman, M.L., Garcia, P.D., Cejka, P., Friedman, K.L., Kowalczykowski, S.C., and Zakian, V.A. Paeschke, K., Bochman, M.L., Garcia, P.D., Cejka, P., Friedman, K.L., Kowalczykowski, S.C., and Zakian, V.A. (2013) Pif1 family helicases suppress genome instability at G-quadruplex motifs. *Nature*, **497**.
19. Schulz, V.P. and Zakian, V.A. Schulz, V.P. and Zakian, V.A. (1994) The *Saccharomyces* PIF1 DNA helicase inhibits telomere elongation and de novo telomere formation. *Cell*, **76**, 145–155.
20. Boulé, J.-B., Vega, L.R., and Zakian, V.A. Boulé, J.-B., Vega, L.R., and Zakian, V.A. (2005) The yeast Pif1p helicase removes telomerase from telomeric DNA. *Nature*, **438**, 57–61.
21. Mateyak, M.K. and Zakian, V.A. Mateyak, M.K. and Zakian, V.A. (2006) Human PIF helicase is cell cycle regulated and associates with telomerase. *Cell Cycle*, **5**, 2796–2804.
22. Boulé, J.-B. and Zakian, V.A. Boulé, J.-B. and Zakian, V.A. (2007) The yeast Pif1p DNA helicase preferentially unwinds RNA DNA substrates. *Nucleic Acids Res.*, **35**, 5809–5818.
23. Eugster, A., Lanzuolo, C., Bonneton, M., Luciano, P., Pollice, A., Pulitzer, J.F., Stegberg, E., Berthiau, A.-S., Förstemann, K., and Corda, Y. *et al.* Eugster, A., Lanzuolo, C., Bonneton, M., Luciano, P., Pollice, A., Pulitzer, J.F., Stegberg, E., Berthiau, A.-S., Förstemann, K., and Corda, Y. (2006) The finger subdomain of yeast telomerase cooperates with Pif1p to limit telomere elongation. *Nat. Struct. Mol. Biol.*, **13**, 734–739.
24. Zhou, J., Hidaka, K., and Futcher, B. Zhou, J., Hidaka, K., and Futcher, B. (2000) The Est1 subunit of yeast telomerase binds the Tlc1 telomerase RNA. *Mol. Cell. Biol.*, **20**, 1947–1955.
25. Puig, O., Caspary, F., Rigaut, G., Rutz, B., Bouveret, E., Bragado-Nilsson, E., Wilm, M., and Séraphin, B. Puig, O., Caspary, F., Rigaut, G., Rutz, B., Bouveret, E., Bragado-Nilsson, E., Wilm, M., and Séraphin, B. (2001) The tandem affinity purification (TAP) method: a general procedure of protein complex purification. *Methods*, **24**, 218–229.
26. Fan, H.-F., Cox, M.M., and Li, H.-W. Fan, H.-F., Cox, M.M., and Li, H.-W. (2011) Developing single-molecule TPM experiments for direct observation of successful RecA-mediated strand exchange reaction. *PLOS ONE*, **6**, e21359.
27. Autexier, C. and Lue, N.F. Autexier, C. and Lue, N.F. (2006) The structure and function of telomerase reverse transcriptase. *Annu. Rev. Biochem.*, **75**, 493–517.
28. Cohn, M. and Blackburn, E. Cohn, M. and Blackburn, E. (1995) Telomerase in yeast. *Science*, **269**, 396–400.
29. Hsu, M. and Lue, N.F. Hsu, M. and Lue, N.F. (2011) Telomeres and telomerase. *Methods Mol. Biol.*, **735**, 97–106.
30. Lin, J.-J. and Zakian, V.A. Lin, J.-J. and Zakian, V.A. (1995) An in vitro assay for *Saccharomyces* telomerase. *Cell*, **81**, 1127–1135.
31. Prescott, J. and Blackburn, E.H. Prescott, J. and Blackburn, E.H. (1997) Functionally interacting telomerase RNAs in the yeast telomerase complex. *Genes Dev.*, **11**, 2790–800.
32. Ramanagoudr-Bhojappa, R., Chib, S., Byrd, A.K., Aarattuthodiyil, S., Pandey, M., Patel, S.S., and Raney, K.D. Ramanagoudr-Bhojappa, R., Chib, S., Byrd, A.K., Aarattuthodiyil, S., Pandey, M., Patel, S.S., and Raney, K.D. (2013) Yeast Pif1 helicase exhibits a one base pair stepping mechanism for unwinding duplex DNA. *J. Biol. Chem.*, **288**.
33. Galletto, R. and Tomko, E.J. Galletto, R. and Tomko, E.J. (2013) Translocation of *Saccharomyces cerevisiae* Pif1 helicase monomers on single-stranded DNA. *Nucleic Acids Res.*, **41**, 4613–4627.
34. Barranco-Medina, S. and Galletto, R. Barranco-Medina, S. and Galletto, R. (2010) DNA binding induces dimerization of *Saccharomyces cerevisiae* Pif1. *Biochemistry*, **49**, 8445–8454.
35. Ha, T., Kozlov, A.G., and Lohman, T.M. Ha, T., Kozlov, A.G., and Lohman, T.M. (2012) Single-molecule views of protein movement on single-stranded DNA. *Annu. Rev. Biophys.*, **41**, 295–319.
36. Chung, C. and Li, H.-W. Chung, C. and Li, H.-W. (2013) Direct observation of RecBCD helicase as single-stranded DNA translocases. *J. Am. Chem. Soc.*, **135**, 8920–8925.
37. Finkelstein, I.J., Visnapuu, M.-L., and Greene, E.C. Finkelstein, I.J., Visnapuu, M.-L., and Greene, E.C. (2010) Single-molecule imaging reveals mechanisms of protein disruption by a DNA translocase. *Nature*, **468**, 983–987.
38. Byrd, A.K. and Raney, K.D. Byrd, A.K. and Raney, K.D. (2004) Protein displacement by an assembly of helicase molecules aligned along single-stranded DNA. *Nat. Struct. Mol. Biol.*, **11**, 531–538.
39. Guy, C.P., Atkinson, J., Gupta, M.K., Mahdi, A.A., Gwynn, E.J., Rudolph, C.J., Moon, P.B., Van Knippenberg, I.C., Cadman, C.J., and Dillingham, M.S. *et al.* Guy, C.P., Atkinson, J., Gupta, M.K., Mahdi, A.A., Gwynn, E.J., Rudolph, C.J., Moon, P.B., Van Knippenberg, I.C., Cadman, C.J., and Dillingham, M.S. (2009) Rep provides a second motor at the replisome to promote duplication of protein-bound DNA. *Mol. Cell*, **36**, 654–666.
40. Byrd, A.K. and Raney, K.D. Byrd, A.K. and Raney, K.D. (2006) Displacement of a DNA binding protein by Dda helicase. *Nucleic Acids Res.*, **34**, 3020–3029.
41. Smith, S., Banerjee, S., Rilo, R., and Myung, K. Smith, S., Banerjee, S., Rilo, R., and Myung, K. (2008) Dynamic regulation of single-stranded telomeres in *Saccharomyces cerevisiae*. *Genetics*, **178**, 693–701.
42. Vega, L.R., Phillips, J.A., Thornton, B.R., Benanti, J.A., Onigbanjo, M.T., Toczyski, D.P., and Zakian, V.A. Vega, L.R., Phillips, J.A., Thornton, B.R., Benanti, J.A., Onigbanjo, M.T., Toczyski, D.P., and Zakian, V.A. (2007) Sensitivity of yeast strains with long G-tails to levels of telomere-bound telomerase. *PLoS Genet*, **3**, e105.
43. Pike, J.E., Burgers, P.M.J., Campbell, J.L., and Bambara, R.A. Pike, J.E., Burgers, P.M.J., Campbell, J.L., and Bambara, R.A. (2009) Pif1 helicase lengthens some Okazaki fragment flaps necessitating Dna2 nuclease/helicase action in the two-nuclease processing pathway. *J. Biol. Chem.*, **284**, 25170–25180.