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Brief Communication

The Pif1 family helicase Pfh1 facilitates telomere replication and has an RPA-dependent role during telomere lengthening

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ABSTRACT

Pif1 family helicases are evolutionary conserved 5′–3′ DNA helicases. Pfh1, the sole *Schizosaccharomyces pombe* Pif1 family DNA helicase, is essential for maintenance of both nuclear and mitochondrial DNAs. Here we show that its nuclear functions include roles in telomere replication and telomerase action. Pfh1 promoted semi-conservative replication through telomeric DNA, as replication forks moved more slowly through telomeres when Pfh1 levels were reduced. Unlike other organisms, *S. pombe* cells overexpressing Pfh1 displayed markedly longer telomeres. Because this lengthening occurred in the absence of homologous recombination but not in a replication protein A mutant (*rad11-D223Y*) that has defects in telomerase function, it is probably telomerase-mediated. The effects of Pfh1 on telomere replication and telomere length are likely direct as Pfh1 exhibited high telomere binding in cells expressing endogenous levels of Pfh1. These findings argue that Pfh1 is a positive regulator of telomere length and telomere replication.

1. Introduction

Telomeres, the DNA–protein structures at the ends of eukaryotic chromosomes, are critical for genome stability. Telomeres in the fission yeast *Schizosaccharomyces pombe*, like their human counterparts, are assembled into a six-membered protein complex called shelterin that protects them from degradation and end-to-end fusions [1]. The *S. pombe* shelterin consists of Pot1, the sequence specific telomere single-strand binding protein, Taz1, the sequence specific duplex DNA binding protein, Poz1, Ccq1, Rap1, and Tpz1 [1,2].

Telomeres pose several problems for DNA replication. Conventional DNA polymerases cannot replicate the very ends of linear chromosomes. In virtually all eukaryotes, this problem is solved by telomerase, a telomere dedicated reverse transcriptase that uses its RNA component as a template to lengthen the G-strand of telomeric DNA. The *S. pombe* telomerase consists minimally of a catalytic subunit Trt1, the templating RNA subunit, TER1 and an accessory subunit, Est1 [3–6]. Although telomerase is critical for telomere maintenance, in *S. pombe*, telomerase deficient cells can survive by either chromosome circularization or Rhp51-dependent homologous recombination (ALT, alternative lengthening of telomeres) [7].

Conventional DNA polymerases also have problems during semi-conservative replication of telomeres. In both budding and fission yeast, replication forks move slowly through telomeric DNA positioned at the end or internally on the chromosome, even in wild type cells [8,9]. In *S. pombe* and mouse, loss of the duplex telomere binding proteins Taz1 (*S. pombe*) or TRF1 (mouse) exacerbates problems in telomere replication [9,10]. In multiple organisms, including humans, chromosomes end in t-loops, which are formed by invasion of the single-stranded G-rich tail of the telomere into duplex telomeric DNA [11]. Although t-loops have not been detected at *S. pombe* telomeres, incubation of 3′ tailed duplex *S. pombe* telomeric DNA with Taz1 generates t-loop structures *in vitro* [12]. T-loops are another challenge to the replication machinery. Taken together, these data suggest that telomeres are hard-to-replicate owing to both their non-nucleosomal protein structure and to the repetitive and G-rich nature of telomeric DNA.

Here we determine if the *S. pombe* Pfh1 DNA helicase, a member of the Pif1 family of 5′–3′ DNA helicases, affects telomeres [13,14]. Unlike budding yeast, which encodes two Pif1 helicases, ScPif1 and ScRrm3 (Sc, *Saccharomyces cerevisiae*), most eukaryotes, including *S. pombe* and humans encode a single Pif1 family helicase, named, respectively, Pfh1 and hPIF1. The three yeast Pif1 family helicases are multifunctional, with critical roles in maintenance of both

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nuclear and mitochondrial DNA [14]. In *S. pombe*, Pfh1 is encoded by an essential gene, and the absence of either the nuclear or the mitochondrial isoform is lethal [15]. Pfh1 facilitates fork progression at many nuclear sites, including highly transcribed RNA polymerase II and III genes, the mating type locus, the rDNA, and converged replication forks [16,17]. Mutations in hPIF1 are found in families with high risk of breast cancer, and *S. pombe* cells with the corresponding mutation are not viable [18]. However, the effect of hPIF1 loss on telomere replication is not resolved [19].

So far, all tested eukaryotic Pif1 family helicases function at telomeres. ScPif1 is a negative regulator of telomere length and telomere addition at double-strand breaks that acts by displacing telomerase from DNA ends [20–23]. Its overexpression results in short telomeres [22], as does overexpression of hPIF1 in human tissue culture cells [24]. In addition, hPIF1 suppresses the long telomere phenotype of *pif1* budding yeast cells [25]. Although ScRrm3 does not inhibit telomerase, it promotes fork progression through telomeric DNA [8].

To understand the telomere functions of Pif1 helicases in an organism that expresses only one Pif1 helicase we examined the role of Pfh1 in *S. pombe* telomere replication. We find that Pfh1 was needed to facilitate fork progression at telomeric repeats, and that this effect is probably direct because telomeres had high Pfh1 association. To resolve conflicting results on the effects of Pfh1 on telomere length, we overexpressed Pfh1, which resulted in telomere lengthening, even in recombination deficient cells, but not in a RPA mutant that has telomerase defects. Thus, Pfh1 is a positive regulator of semi-conservative telomeric DNA replication and performs a unique PIF1 family function in telomerase-mediated telomere lengthening.

2. Results and discussion

2.1. Pfh1 facilitates replication fork progression through telomeres

Pfh1 promotes replication through multiple types of hard-to replicate sites [16,17]. As *S. pombe* telomeric DNA impedes replication fork progression even in wild type (WT) cells [9], we asked if Pfh1 also affects semi-conservative replication at telomeres. To do so, we examined telomere replication intermediates in a strain (YSA60; Table S1) where Pfh1 was expressed as a GFP fusion under the control of the thiamine-repressible *nmt81* promoter (*nmt81-pfh1-GFP*). The Pfh1-GFP fusion was expressed from its endogenous locus (Fig. S1).

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To visualize replication intermediates, we used two-dimensional (2D) gel electrophoresis (Fig. 1A–E). DNA from log phase cells was digested with EcoRV, which in our strain liberated three telomere fragments [9] (Fig. 1B). As shown previously, in WT cells, replication forks slowed as they moved through telomeric DNA as reflected by the increased intensity of telomeric replication intermediates relative to other sequences in sub-telomeric DNA [9] (Fig. 1C, see arrows). DNA was prepared from otherwise isogenic *nmt81-pfh1-GFP* expressing cells and examined by 2D gel analysis after 12 h of growth in thiamine, when Pfh1 is no longer detected by western blot analysis [15,16]. Replication fork pausing within telomeric DNA was three- to four-fold higher in Pfh1-depleted compared to isogenic Pfh1 expressing cells (Fig. 1C–E, see arrows). Thus, Pfh1 promotes fork progression through duplex telomeric DNA.

2.2. Pfh1 associates with telomeric DNA in vivo

Using Co-IP, mass spectrometry and ChIP-Seq we found that Pfh1 is a replisome component, which interacts with all nuclear sequences at their time of replication (Sabouri et al. in preparation). However, if Pfh1 has a direct effect on telomere replication, it is possible that its telomere association will be higher than at other genomic sites.

Pfh1 binding to telomeres was assessed using ChIP combined with quantitative PCR (qPCR). We used a strain expressing epitope-tagged Pfh1-13Myc expressed from the *leu1⁺* locus under control of the *pfh1⁺* promoter (the endogenous *pfh1⁺* locus was not modified) (YNS29; Table S1 and Fig. S1) [16]. As a control, we used an otherwise isogenic strain that expressed untagged Pfh1 from its endogenous locus (No tag; Fig. S1, wild type). In both strains, we compared Pfh1 binding to the sub-telomeric STE sequence (STE) to its binding to the *gal1⁺* gene (gal1). Although Pfh1 binding to *gal1⁺* was significantly higher than the no tag control (as expected for a replisome component), Pfh1 association was ~25-fold higher at telomeres than at *gal1⁺* (Fig. 1F). Thus, Pfh1 is telomere associated *in vivo* to a much greater extent than can be explained by its association with the replisome.

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2.3. Pfh1 is a positive regulator of telomere length

To re-examine the effects of Pfh1 on telomere length, we overexpressed it using a multi-copy plasmid, pVS117 (*pfh1⁺* plasmid). Overexpression was verified by western blot analysis (Fig. 2B and Fig. S1), which showed that levels of Pfh1 were at least four times higher in cells with the Pfh1 overexpressing plasmid compared to cells without the plasmid. The plasmid was introduced into a strain that expresses only Pfh1-mt* (YSP377; Table S1), a version of Pfh1 that is targeted almost exclusively to mitochondria, although it maintains enough nuclear Pfh1 to maintain viability at 30 °C [15]. Telomere length was assessed by Southern blot analysis of ApaI-digested DNA (Fig. 2A).

Cells overexpressing Pfh1 exhibited telomere lengthening. After 25 generations, telomeres in Pfh1 overexpressing cells were about 450 bps in length (i.e., ~150 bp longer than WT telomeres), and this length was maintained for multiple restreaks (Fig. 2C, compare lane 1 to lanes 2–10). Upon loss of the Pfh1 overexpression plasmid, telomeres returned to WT lengths (Fig. 2C, lanes 11–17). When the cells that had lost the plasmid were retransformed with the Pfh1 plasmid, telomeres again elongated (Fig. 2C, lanes 18–25). In contrast, cells retransformed with an empty vector had wild-type telomere length (Fig. 2C, lanes 26–34). Thus, overexpression of Pfh1 results in reversible telomere lengthening suggesting that Pfh1 is a positive regulator of telomere length.

2.4. Pfh1-induced telomere lengthening occurs in the absence of Rph51

Overexpression of Pfh1 could promote telomere lengthening by stimulating telomerase or by promoting recombination, as occurs in some cells lacking telomerase [7]. The ALT pathway is Rhp51-dependent [7], as is virtually all homologous recombination in *S. pombe*. When Pfh1 was overexpressed in *rhp51Δ* cells, telomeres still lengthened (Fig. 3A, lanes 4–6). However, lengthening did not occur in *rhp51Δ* cells carrying the empty vector (Fig. 3A, lanes 1–3) or no vector (Fig. 3A, lanes 10–12). The effect on telomere length required the helicase activity of Pfh1 as overexpressing a helicase dead variant, Pfh1-K388A, in which the invariant lysine in the Walker A box was mutated to alanine, did not result in