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Pif1p Helicase, a Catalytic Inhibitor of Telomerase in Yeast

J.-Q. Zhou, E. K. Monson,* S. -C. Teng,* V. P. Schulz,*† V. A. Zakian‡

Mutations in the yeast *Saccharomyces cerevisiae PIF1* gene, which encodes a 5′-to-3′ DNA helicase, cause telomere lengthening and a large increase in the formation rate of new telomeres. Here, we show that Pif1p acts by inhibiting telomerase rather than telomere-telomere recombination, and this inhibition requires the helicase activity of Pif1p. Overexpression of enzymatically active Pif1p causes telomere shortening. Thus, Pif1p is a catalytic inhibitor of telomerase-mediated telomere lengthening. Because Pif1p is associated with telomeric DNA in vivo, its effects on telomeres are likely direct. Pif1p-like helicases are found in diverse organisms, including humans. We propose that Pif1p-mediated inhibition of telomerase promotes genetic stability by suppressing telomerase-mediated healing of double-strand breaks.

PIF1 is a nonessential *Saccharomyces* gene that encodes a 5′-to-3′ DNA helicase (*1*). Mutations in *PIF1* affect telomeres in three ways: telomeres from *pif1* mutant cells are longer than wild-type telomeres; healing of double-strand breaks by telomere addition occurs much more often in *pif1* cells than in wild-type cells; and *pif1* cells but not wild-type cells add telomeric DNA to sites that have very little resemblance to telomeric DNA (*2*). These data suggest that Pif1p is an inhibitor of telomere lengthening. Pif1p also affects mitochondrial (*1*) and ribosomal DNA (*3*).

There are two mechanisms that can lengthen the ~300–base pair (bp) tracts of yeast telomeric $C_{1\text{-}3}A/TG_{1\text{-}3}$ DNA: telomerase (*4*) and telomere-telomere recombination (*5*). In the absence of genes required for telomerase such as *TLC1*, which encodes telomerase RNA (*6*), and *EST1*, which encodes a telomerase RNA binding protein (*7*), telomeric DNA gets shorter and shorter, the cultures senesce, and most cells eventually die. Lengthening of telomeres by recombination requires the continued presence of Rad52p (*5*). If Pif1p inhibits telomere-telomere recombination, telomere lengthening will not occur in a *pif1 rad52* strain, and a *pif1 tlc1* (or *est1*) strain might not senesce or would senesce more slowly due to activation of the recombinational pathway for telomere maintenance. If Pif1p inhibits telomerase, telomere lengthening would not occur in *pif1 tlc1* or *pif1 est1* strains. To distinguish between these possibilities, we constructed singly and doubly mutant strains of the appropriate genotypes and examined telomere lengths (*8*). Because telomeres were at least as long in a *pif1 rad52* as in a *pif1* strain (Fig. 1A), the effects of Pif1p did not require Rad52p. In contrast, telomere lengthening did not occur in either a *pif1 tlc1* (Fig. 1B) or a *pif1 est1* strain (*9*). In addition, lack of Pif1p did not bypass or even delay the senescence phenotype of cells lacking telomerase (Fig. 1C). Thus, Pif1p inhibits a telomerase-dependent pathway of telomere lengthening.

To determine if the helicase function of Pif1p is required to inhibit telomere lengthening, we used site-directed mutagenesis to modify the invariant lysine in the ATP-binding domain to either alanine (K264A) or arginine (K264R) (*10*), as this residue is essential for the activity of other helicases (*11*). Both the wild-type and the K264A mutant version of Pif1p were expressed in Sf9 insect cells infected with recombinant baculovirus, purified to near homogeneity (Fig. 2A), and their activities assayed in vitro. Whereas wild-type Pif1p catalyzed unwinding of a 17-base, [^{32}P]end-radiolabeled oligonucleotide annealed to a M13 single-strand circle, the K264A allele had no helicase activity in this assay (Fig. 2B).

To determine the phenotype of cells that lacked Pif1p helicase activity, strains with only the K264A or K264R allele were constructed (*12*). DNA was prepared from cells carrying these mutant alleles, as well as from

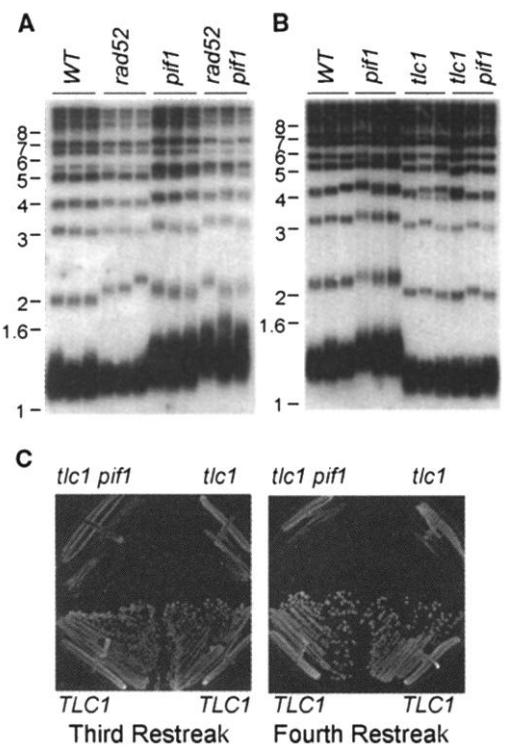

Fig. 1. Pif1p inhibits telomerase, not telomere-telomere recombination. **(A)** DNA was prepared from three independent transformants from otherwise isogenic strains of the indicated genotypes. The DNA was digested with Xho I and analyzed by Southern hybridization using a $C_{1\text{-}3}A/TG_{1\text{-}3}$ telomeric probe here and in (B). The *pif1-m2* allele, which affects telomeric but not mitochondrial DNA (*2*), was used here and in (B) and (C). **(B)** A diploid strain heterozygous at both *TLC1* and *PIF1* was sporulated, tetrads were dissected, and the genotype of the spore products was determined. DNA was isolated from independent spores with the indicated genotypes ~30 cell divisions after sporulation. **(C)** Individual spores from tetrads obtained as in (B) were streaked on rich medium and grown to single colonies (~25 cell divisions). Individual colonies were restreaked repeatedly. The third and fourth restreaks after sporulation are shown for the four spore products from one of nine tetrads examined.

Department of Molecular Biology, Princeton University, Princeton, NJ 08544–1014, USA.

*These authors contributed equally to this work.
†Present address: Genaissance Pharmaceuticals, Five Science Park, New Haven, CT 06511, USA.
‡To whom correspondence should be addressed: E-mail: vzakian@molbio.princeton.edu

the wild-type *pif1Δ*, *pif1-m1*, or *pif1-m2* strains, and examined by Southern analysis (Fig. 2C). The *pif1-m1* and *pif1-m2* alleles are point mutations in, respectively, the first or second AUG of the *PIF1* open reading frame (ORF): *pif1-m1* cells have mutant mitochondrial function but wild-type telomeres, whereas *pif1-m2* cells have wild-type mitochondria but mutant telomeres (*2*). As expected, *pif1Δ* (Fig. 2C, lane 2) and *pif1-m2* (lane 4) cells had long telomeres, whereas *pif1-m1* cells (lane 3) had telomeres of wild-type length (lanes 1 and 9). The strains with the K264A and K264R alleles also had long telomeres (lanes 5 and 6). That this telomere lengthening was due to the point mutations in the ATP-binding pocket was demonstrated by restoration of wild-type telomere length in K264A and K264R cells carrying a plasmid-borne copy of wild-type *PIF1* (lanes 7 and 8).

Western analysis established that cells carrying the K264A and K264R alleles produced stable Pif1p (Fig. 2D). Wild-type cells contained two similarly sized proteins of ~94 kD, the expected size for Pif1p (lane 1). The mitochondrially defective *pif1-m1* strain expressed only the longer form of Pif1p (lane 3), whereas the telomere-impaired *pif1-m2* strain expressed only the faster migrating species (lane 4). These data provide direct evidence for the hypothesis (*2*) that Pif1p is targeted to different subcellular compartments by making two forms of the protein, one localized to mitochondria and the other, whose translation begins at the second AUG in the ORF, destined for the nucleus. (The mitochondrial Pif1p was shorter than nuclear Pif1p due to proteolysis during import into mitochondria). Because the K264A and K264R alleles produced levels of the nuclear form of Pif1p similar to those in the wild type (lanes 5 and 6), their effects on telomere length must be due to loss of Pif1p adenosine triphosphatase/helicase activity.

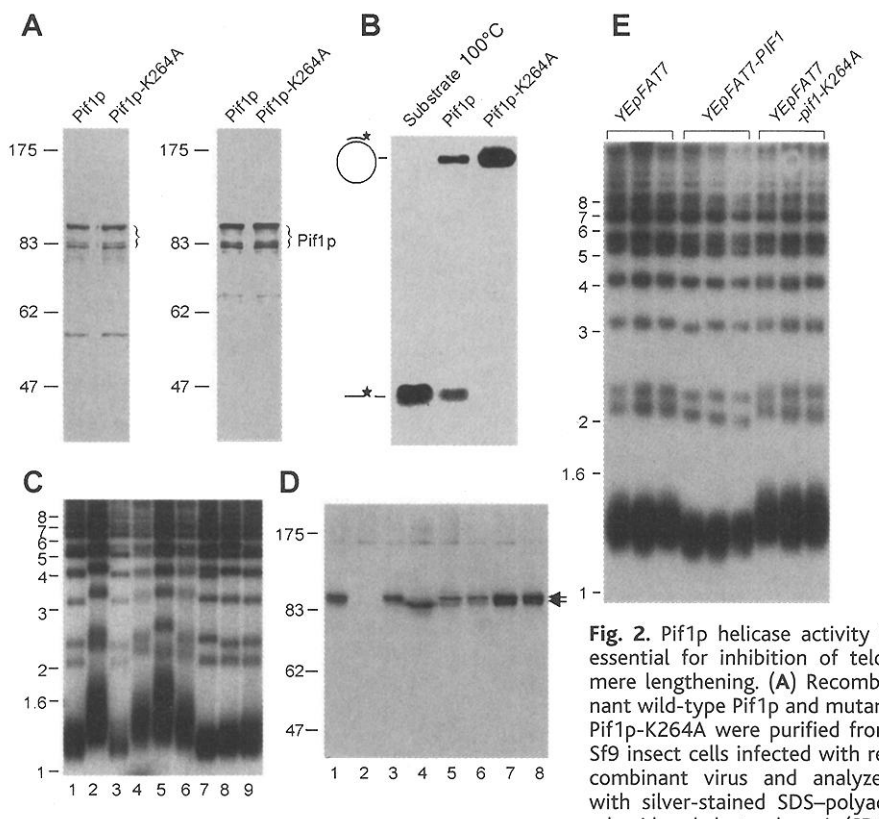

Fig. 2. Pif1p helicase activity is essential for inhibition of telomere lengthening. (**A**) Recombinant wild-type Pif1p and mutant Pif1p-K264A were purified from Sf9 insect cells infected with recombinant virus and analyzed with silver-stained SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (left panel) or Western blotting with affinity-purified antibodies to Pif1p (right panel). (**B**) Helicase activity assays were carried out using a 1-μM solution of the partial duplex DNA substrate (a 5′, [^{32}P]end-radiolabeled, 17-nucleotide oligomer annealed to the single-stranded M13mp7 DNA) and 50 ng of purified recombinant protein from (A). Reactions were incubated at 37°C for 30 min and analyzed by 10% PAGE and autoradiography. (**C**) Genomic DNA from wild-type and mutant strains was analyzed as in Fig. 1A. The lanes contain DNA from otherwise isogenic wild-type (lane 1), *pif1Δ* (lane 2), *pif1-m1* (lane 3), *pif1-m2* (lane 4), *pif1-K264A* (lane 5), *pif1-K264R* (lane 6), *pif1-K264A* carrying pVS102, a centromere plasmid bearing wild-type *PIF1* (lane 7), *pif1-K264R* carrying pVS102 (lane 8), and the wild type (lane 9). (**D**) Western analysis was performed on proteins isolated from the mutant and wild-type cells whose DNA was examined in (C), using affinity-purified anti-Pif1p serum. Lanes are the same as in (C). (**E**) Genomic DNA was isolated from three independent cultures of *PIF1* cells carrying either the multicopy vector YEpFAT7, YEpFAT7 containing the *PIF1* gene, or YEpFAT7 containing the *PIF1*-K264A mutant gene, and then analyzed by Southern blotting as in Fig. 1A.

If Pif1p is an inhibitor of telomerase, overexpression of Pif1p might result in telomere shortening. To test this possibility, *PIF1* was cloned into the multicopy YEpFAT7 plasmid (*13*). *PIF1* cells carrying YEpFAT7-*PIF1* had telomeres that were ~80 bp shorter than cells carrying YEpFAT7 (Fig. 2E). In contrast, wild-type cells carrying YEpFAT7-K264A *PIF1* did not show telomere shortening. The fact that overexpression of wild-type but not helicase-deficient Pif1p caused telomere shortening is consistent with the idea that Pif1p acts enzymatically to inhibit telomerase.

Mutations in certain essential replication proteins also cause telomere lengthening when cells are grown at semipermissive temperatures for loss of function alleles (*14*). Analysis by fluorescence-activated cell sorting (FACS) shows that the replication mutants with lengthened telomeres had an abnormally large number of S-phase cells. Because telomeric DNA is replicated at the end of S phase (*15*), changes in telomere length in these mutants might be linked to their genome-wide slowing of DNA replication. In contrast, the FACS profile of *pif1* cells revealed that they had a similar fraction of S-phase cells as the isogenic wild-type strain (Fig. 3A). In addition, cells lacking Pif1p have wild-type rates of chromosome loss and mitotic recombination (*2*), whereas mutants in general replication proteins increase both (*16*). These data suggest that Pif1p has a direct effect on telomere replication.

If Pif1p acts directly to inhibit telomere replication, it should associate physically with telomeric DNA. To assess this possibility, chromatin was cross-linked in vivo with formaldehyde, sheared to ~1000 bp, and then precipitated with either protein A–purified preimmune antibodies [rabbit immunoglobulin G (IgG)] or affinity-purified anti-Pif1p antibodies (α-Pif1p) (Fig. 3B). As a positive control, chromatin was also precipitated with an anti-Rap1p serum (α-Rap1p): Rap1p, the major structural protein at yeast telomeres is constitutively bound to telomeric DNA (*17*). The cross-links in the immunoprecipitate were reversed, and the DNA in the immunoprecipitate was amplified with polymerase chain reaction (PCR) using primers that amplified a 233-bp portion of the subtelomeric Y′ element that lies 30 bp upstream of the start of the telomeric repeats or, as a negative control, primers for a 131-bp fragment of the *ACT1* gene. Both the anti-Rap1p and anti-Pif1p serum specifically precipitated telomeric DNA. Twofold serial dilutions of immunoprecipitates revealed that telomeric DNA was enriched 5.0 ± 2.0 fold (mean ± SD) in the anti-Pif1p precipitate compared to its presence when preimmune antibodies were used or when the anti-Pif1p antibodies were used to precipitate chromatin from *pif1Δ* cells. As Pif1p was not telomere asso-

ciated in the absence of cross-linking (X-link –), its association must have occurred in vivo.

When the sequence of the 859–amino acid Pif1p was compared to the translated DNA data base, several highly similar genes were detected including a second *S. cerevisiae* gene called *RRM3* (Fig. 4A). None of the other 132 yeast ORFs with helicase motifs (*18*) had detectable similarity to Pif1p by the criterion of a blast search (*19*). This search also identified Pif1p-like proteins in *Candida maltosa*, *Caenorhabditis elegans*, and *Drosophila melanogaster* (Fig. 4A). We isolated *PIF1*-like genes from both *Schizosaccharomyces pombe* (*rph1*$^+$, *RRM3*/*PIF1* homolog) and *Homo sapiens* (hPif1p, human Pif1p) (*20*). Thus, Pif1p is the prototype member of a helicase subfamily, conserved from yeasts to humans. Pif1 subfamily members encode proteins with 30 to 50% identity in all pairwise combinations over a region of more than 300 amino acids (Fig. 4A). This degree of relatedness is similar to or greater than that seen within other helicase subfamilies (*21*). Because all of the Pif1p-like proteins had very high identity to Pif1p, a known DNA helicase, within each of the helicase motif regions (*22*), the other members of the Pif1p subfamily are also likely to be helicases. Like Pif1p, the *Saccharomyces* Rrm3p and the *S. pombe* Rph1p also affect telomeric DNA (*19*).

We demonstrate that the *Saccharomyces* Pif1p inhibits telomerase lengthening of telomeric DNA (Fig. 1). Telomere length was inversely proportional to the amount of Pif1p in cells. Loss of Pif1p led to telomere lengthening (Fig. 2C), and overexpression of Pif1p caused telomere shortening (Fig. 2E). The catalytic activity of Pif1p was required for both of these effects (Fig. 2, C and E). Although helicases are required for transcription, RNA processing, and translation, as well as for DNA replication, the association of Pif1p with telomeric DNA in vivo (Fig. 3B) argues strongly that its effects on telomeres are direct.

How might a 5′-to-3′ DNA helicase counter telomerase activity? Because yeast chromosomes have 3′ single-strand tails (*23*), Pif1p does not have the right polarity to unwind chromosomes from their ends (Fig. 4B, left). However, Pif1p could dissociate the last Okazaki fragment to generate a long single-strand $TG_{1\text{-}3}$ tail (Fig. 4B, middle). Although a priori, G-tails seem more likely to stimulate than inhibit telomerase, $TG_{1\text{-}3}$ tails might fold into a structure that prevents telomerase lengthening (*24, 25*). Alternatively, the helicase activity of Pif1p might dissociate telomeric DNA from telomerase RNA (Fig. 4B, right). If Pif1p preferentially dissociates RNA-DNA hybrids held together by a very small number of base pairs, it would explain the reduced specificity of telomere addition seen in the absence of Pif1p (*2*).

Is there an advantage to inhibiting telomerase? Cells lacking the nuclear form of Pif1p had no cell-cycle defect (Fig. 3A) and wild-type chromosome stability (*2*). Thus, Pif1p is not important in the normal mitotic cell cycle. However, Pif1p might be critical after DNA damage. When a yeast chromosome loses a telomere, the broken chromosome is either lost or a new telomere is gained by homologous recombination (*26*). Although wild-type cells very rarely add telomeres de novo to broken chromosomes (*2, 27*), the rate of de novo telomere addition in

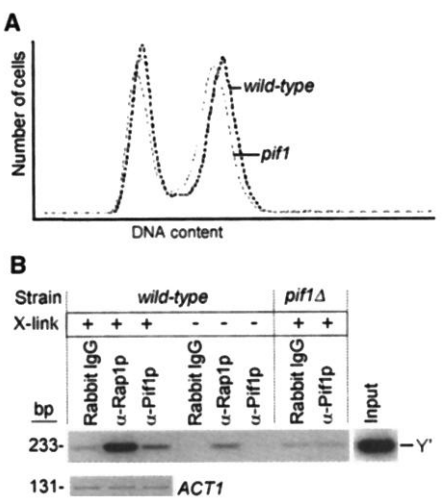

Fig. 3. Pif1p affects telomeric DNA directly. (**A**) Log phase *pif1-m2* and otherwise isogenic wild-type cells were grown in rich medium, fixed, stained with propidium iodide, and analyzed by fluorescence activated cell sorting (FACS). (**B**) Chromatin was prepared from otherwise isogenic wild-type or *pif1Δ* cells that had been cross-linked (X-link +) or not (X-link –) with formaldehyde in vivo. Immunoprecipitation was carried out as described in (*3*) using either protein A–purified preimmune IgG (rabbit IgG), a polyclonal Rap1p antiserum (α-Rap1p) (*17*), or affinity purified anti-Pif1p polyclonal antibodies (α-Pif1p). The DNA in the immunoprecipitate was PCR-amplified for 28 cycles using telomeric primers or for 32 cycles using *ACT1* primers, separated in an agarose gel, and visualized by staining with ethidium bromide. PCR amplification of the input DNA with telomeric primers is also shown. Although Pif1p association with telomeric DNA did not occur in the absence of cross-linking, the amount of telomeric DNA precipitated with anti-Rap1p was not eliminated in non–cross linked cells.

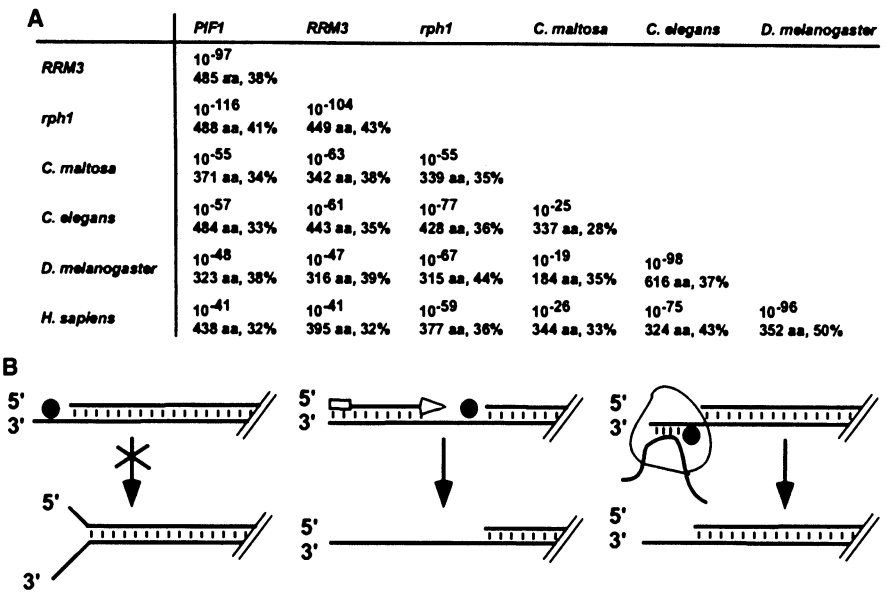

Fig. 4. *PIF1* is the prototype of a subfamily of putative helicases. (**A**) The predicted sequences of the *PIF1*-like proteins were compared using the TBLASTN 2.06 program (*29*). The top line for each pair shows the expectation value, a measure of the probability that the match occurred by chance. The number of amino acids of homology shared between the two proteins and percentage identity within the helicase region is shown below. These numbers were obtained by aligning the helicase region using the MacVector 6.0 (Oxford Molecular) implementation of the ClustalW program (*30*). (**B**) Models for Pif1p inhibition of telomerase lengthening. The left telomere of a single chromosome is shown. The solid circle is Pif1p. (Left) A 3′-to-5′ helicase could unwind chromosomes from their ends to create a substrate for a nuclease that inhibits telomerase by destroying its substrate. Because Pif1p is a 5′-to-3′ helicase, this model can not explain the effects of Pif1p. (Middle) Pif1p might dissociate the last Okazaki fragment to generate a ~100 base $TG_{1\text{-}3}$ tail. This single-strand $TG_{1\text{-}3}$ tail could inhibit telomerase either by forming a higher-order structure, such as a G-quartet (*24*) or t-loop (*25*), or by serving as a substrate for an exo-nuclease. The open rectangle represents the 8- to 12-base RNA that primes Okazaki fragments. (Right) Pif1p might inhibit telomerase directly by promoting dissociation of the telomerase RNA-telomeric DNA hybrid that is an intermediate in telomere replication. The structure base paired to the 3′ single-strand tail is telomerase RNA.

pif1 cells is elevated as much as 600-fold (*2*). Adding a telomere to a double-strand break results in aneuploidy for sequences distal to the site of telomere addition. By inhibiting such events, Pif1p could promote genetic stability.

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8. *Saccharomyces cerevisiae* strains isogenic to VPS106 were used (Fig. 1) (*2*). *RAD52* was inactivated by transforming cells with a fragment containing *LEU2* inserted at the unique Bgl II site within the coding region of *RAD52*. The *TLC1* gene was inactivated by transforming diploid VPS107 *PIF1/pif1-m2* cells with Xho I–digested pBlue61::LEU2, which deletes 693 bp from the *TLC1* coding region (*6*). The resulting diploid strain, VPS107*PIF1/pif1-m2 TLC1/tlc1::LEU2*, was sporulated, and tetrads were dissected. Individual spore products from nine tetrads were streaked four times on rich medium and examined at each streak for growth rate and viable colonies. DNA for analysis of telomere length was prepared ~30 cell divisions after sporulation and ~5 cell divisions after each restreak.
9. J.-Q. Zhou *et al.*, data not shown.
10. Mutagenesis of lysine 264 in *PIF1* to alanine (K264A) or arginine (K264R) was done using the QuickChange Site-directed Mutagenesis Kit (Stratagene) on the 488-bp Afl II–Pfl MI *PIF1* fragment, which was sequenced after mutagenesis to ensure that it contained only the desired mutation. The mutagenized fragment was inserted into pVS102, which contains a genomic Sac I–Apa I *PIF1* fragment, replacing the wild-type portion of the gene. The pVS102 mutant or wild-type *PIF1* plasmids were digested with Sac I–Cla I, and the fragment containing *PIF1* was inserted at the Bam HI site in the pFastBac1 vector (Gibco–BRL). For the purification of recombinant proteins, 1 liter of Sf9 cells at a density of 1.5×10^6/ml were infected at a multiplicity of 10 and harvested 60 hours after infection. The recombinant proteins in the cytoplasmic fraction were followed by Western blotting, using a Pif1p antiserum (*3*), and purified with chromatography on phosphor-cellulose, Q Sepharose, and heparin agarose.
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20. The *S. pombe rph1*$^+$ was isolated using PCR primers that were based on conserved amino acid residues present in both *PIF1* and the *C. maltosa* Pif1p-like protein (accession number AF074944). By comparing the Pif1p sequence with the translated database of expressed sequence tags, we found one human gene (accession number AA464521) with low levels of similarity (expectation value of 0.015). We extended this sequence 3′ to the poly(A) tail by further searching of the human gene against the expressed sequence tag database (unigene number Hs.112160). The raw sequence was corrected by inspection of the automated sequencing traces, and when this sequence was compared to the protein database, high levels of similarity to the *PIF1* subfamily were found. We extended the sequence deduced from the database to 374 amino acids (accession number AF108138) using rapid amplification of cDNA ends. We were unable to obtain the sequence of the NH_2-terminal portion of hPif1 because, after 374 amino acids, its cDNA was fused to an Alu element as a result of template switching during cDNA synthesis.
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Pol κ: A DNA Polymerase Required for Sister Chromatid Cohesion

Zhenghe Wang, Irene B. Castaño,* Alejandro De Las Peñas,* Carrie Adams, Michael F. Christman†

Establishment of cohesion between sister chromatids is coupled to replication fork passage through an unknown mechanism. Here we report that *TRF4*, an evolutionarily conserved gene necessary for chromosome segregation, encodes a DNA polymerase with β-polymerase–like properties. A double mutant in the redundant homologs, *TRF4* and *TRF5*, is unable to complete S phase, whereas a *trf4* single mutant completes a presumably defective S phase that results in a failure of cohesion between the replicated sister chromatids. This suggests that *TRF*s are a key link in the coordination between DNA replication and sister chromatid cohesion. Trf4 and Trf5 represent the fourth class of essential nuclear DNA polymerases (designated DNA polymerase kappa) in *Saccharomyces cerevisiae* and probably in all eukaryotes.

We identified the *TRF4* gene in a genetic screen for functions redundant with DNA topoisomerase I (*1*) and showed that, together with its close homolog, *TRF5*, the genes are essential for several events in DNA metabolism including chromosome segregation (*2*, *3*) and DNA damage repair (*4*). Recent analysis of the highly conserved *TRF4* gene family has led to the conclusion that *TRF*s are members of the β-polymerase superfamily (*5*), which consists of proteins that catalyze a variety of nucleotidyltransferase reactions including DNA synthesis.

To test the prediction that TRFs possess nucleotidyltransferase activity (*5*), we purified Trf4 fused to a six-histidine tag from *Escherichia coli* to apparent homogeneity (*6*, *7*) and assayed recombinant protein for DNA polymerase activity. First, Trf4 was examined for the ability to extend a 5′ end-labeled oligo(dT) primer (16 mer) that was hybridized to a poly(dA) template [average size: 282 nucleotides (nt)] in the presence of deoxythymidine triphosphate (dTTP) and Mg^{2+} (Fig. 1A). Trf4 is able to extend the primer in a distributive manner (extension of a single nucleotide followed by dissociation from primer/template), which is characteristic of β-DNA polymerases (*8*). In contrast, a mutant Trf4 protein missing the NH_2-terminal 240 amino acids of the 584–amino acid protein, Trf4Δ240, was completely unable to polymerize nucleotides (Fig. 1A, lanes 5 to 7).

Fractions eluted from the final purification step, a mono Q anion exchange column, demonstrate cofractionation of Trf4 protein and DNA polymerase activity (Fig. 1B). The

Department of Microbiology, University of Virginia, Box 441, Jordan Hall, 1300 Jefferson Park Avenue, Charlottesville, VA 22908, USA.

*Present address: Johns Hopkins University, Department of Molecular Biology and Genetics, 725 North Wolfe Street, 504 PCTB, Baltimore, MD 21205–2185, USA.

†To whom correspondence should be addressed. E-mail: mfc3f@virginia.edu