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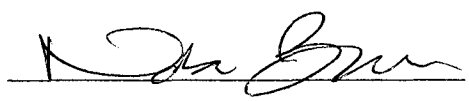

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13. ABSTRACT *(Maximum 200 Words)*

Telomeres are repeated double stranded DNA sequences at chromosome ends. Replication of telomeres requires telomerase. Recent data suggest telomere length maintenance might be involved in the origin or progression of cancer and aging. Understanding of telomere replication could offer new targets for drug screen. The conservation of telomerase and telomere structure has made yeast a good system to study the problem. An essential yeast telomere binding protein Cdc13p appears to play a key regulatory role in telomere replication. This study showed that Pol α is involved in telomere replication. Cdc13p regulates telomere length during telomere replication by interacting with both telomerase and Pol α. This study also identified Cdc13p and yeast PCNA interaction. This interaction might be important for limiting C-strand degradation, process necessary for telomere replication and can kill cells when not limited. Other Cdc13p interacting proteins were also identified. Further studies are in progress.

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1. Introduction:

The mechanism of DNA replication used by all known polymerases predicts one problem: at the end of DNA replication, after removal of the last RNA primer, there will be a loss of 7-12 nucleotides at one 5' end of a newly synthesized chromosome. This loss of DNA is compensated for by telomerase, an reverse transcriptase that uses an RNA template to extend the 3' ends. The conventional DNA polymerase can then repair the 5' ends. Since telomerase RNA templates are C-rich, the 3' ends of chromosomes synthesized by telomerase are G-rich and the strands are called G-strands. The other strands, repaired by a DNA polymerase, are called C-strands. The chromosomal ends containing the repeated sequences are called telomeres. In budding yeast, telomeres are about 300±75 bps, consisting of repeated sequences $TG_{1\text{-}3}/C_{1\text{-}3}A$(Wang et al., 1989; Wang and Zakian, 1990; Zakian, 1995).

Cdc13p is a budding yeast telomere binding protein *in vivo* and *in vitro*(Bourns et al., 1998; Lin and Zakian, 1996; Nugent et al., 1996). It appears to play a central regulatory role in telomere replication. *cdc13-2* mutant cells have the phenotype as a mutant that lack telomerase activity: telomeres shorten progressively until the cells senesce. However, extracts from these cells have normal telomerase activity *in vitro*(Nugent et al., 1996). This suggests that Cdc13p might recruit telomerase activity *in vivo*. The *cdc13-1* mutant is a temperature sensitive mutation. At the restrictive temperature, cells are arrested due to C-strand specific degradation by an unknown mechanism. This degradation starts from the C-strands of telomere ends and can go up to 20 kb into the chromosomes(Garvik et al., 1995). Cdc13p therefore appears to protect the telomeres from degradation. Studying Cdc13p interacting proteins might yield important information about telomere replication and regulation.

2. Body:

Since joining in Dr. Virginia A. Zakian's lab in 1996, I have focused on studying Cdc13p and its interacting partners. Using a two-hybrid assay, I was able to identify proteins that interact with Cdc13p, which include: DNA polymerase α catalytic subunit Pol1p, the essential telomere replication protein Est1p, Tao3p, Fun12p and others.

a. Cdc13p-Est1p and Cdc13p-Pol1p interaction

The Cdc13p-Pol1p two-hybrid interaction was confirmed by a biochemical assay. Pol1p could be co-immunoprecipitated with Cdc13p when both protein were expressed from their own promoters. These data suggest that the two proteins are in the same complex *in vivo*(Qi and Zakian, 2000).

Single-amino-acid mutations that disrupt the Cdc13p-Pol1p interaction were generated by a random PCR mutagenesis assay. The single-amino-acid mutations obtained in Pol1p clustered together: D236N, E238K, P241T. Each amino acid change disrupted the Cdc13p-Pol1p interaction to a different extent: D236N, 100%; E238K,

50%; and P241, 90%. These mutations were placed back into the cells and the mutated alleles have no general DNA replication defect or growth defect, but do have longer telomeres. The extent of the lengthening of the telomeres is correlates with the severity of the loss of the interaction(Qi and Zakian, 2000).

Cdc13p residues involved in Cdc13-Pol1p interaction were also mapped by the same PCR mediated random mutagenesis. They were throughout the protein. Two of the mutations were placed back into the chromosome. These mutations also showed only a long telomere phenotype(Qi and Zakian, 2000).

TPE(Telomere Position Effect on transcription) can be used as an indicator of telomere structure. The mutations described above have no effect on TPE suggesting that overall telomere structure is normal and the defect is restricted to termini(Qi and Zakian, 2000).

The Cdc13p-Est1p two-hybrid interaction was confirmed by biochemical criteria. Cdc13p can be co pulled down by GST beads in cell extracts over-expressing both Cdc13p and GST tagged Est1p. Over-expression of an Est1p fragment partially suppressed the lethality of *cdc13-1*. These data suggest that Cdc13p interacts with Est1p physically and genetically. *cdc13-2* cells appear to lose the ability to recruit telomerase *in vivo*. However cdc13-2p still interacted with Est1p in a two-hybrid assay and a GST-pull-down assay(Qi and Zakian, 2000). We propose that the Cdc13p-Est1p interaction results in a conformation change that is critical for recruiting telomerase activity. The *cdc13-2* phenotype is not due to the loss of the interaction with Est1p, but the inability of the mutant proteins to undergo a conformation change.

The above results suggest: 1. DNA polymerase α is the conventional enzyme involved in C-strand repair during telomere replication. 2. Cdc13p interacts with both the telomerase component Est1p and the C-strand repairing enzyme Pol1p to coordinates telomere synthesis. Two models have been proposed: cooperation and competition, to explain how Cdc13p coordinates G-strand and C- strand synthesis(midterm report).

b. Cdc13p-Fun12p

Fun12p is a translation initiation factor in budding yeast(Choi et al., 1998). It was found to interact with Cdc13p in a two-hybrid screen. Using a PCR mediated random mutagenesis assay, single-amino-acid mutations in Fun12p that disrupted Cdc13p-Fun12p interaction were isolated. They are I634V, S628F, S566F and F843L. The first two were placed back into the *FUN12* locus on chromosome I. Mutants showed no growth defects and no telomere length defects. The meaning of this interaction remains unknown. Further studies are in progress(midterm report).

c. Cdc13p-Tao3p

Tao3p is a potential transcription factor with seven transmembrane domains. A mutant form of Tao3p activates the expression of Och1p, a protein involved in protein N-

linked glycosylation and cell wall synthesis. I found that the C-terminus 90 amino acids of Tao3p (2376aa) interacts with Cdc13p a in two-hybrid assay. Deletion of *TAO3* gene results in cell morphology changes: multi-budded cells, cell aggregation, and slightly short telomeres. Deletion of the Cdc13p interacting C-terminus of Tao3p gives the same phenotype. Furthermore, Cdc13-1p that lost interaction with Tao3p in a two-hybrid assay also generates some multiple budded cells at the permissive temperature. The aggregation of cells can be dissolved by glusulase digestion, suggesting the defect is at mother-daughter cell wall separation. These results suggest that the Cdc13p-Tao3p interaction is important for normal budding and cell morphology.

Homologues of *TAO3* have been found in *S. pombe*, fruit fly, *C. elegans* and human. The conservation suggests a function importance. The human homology of Tao3p (CG003) is upstream of *BRCA2* and the c-terminus of hTAO3 was found deleted in a familial pancreatic carcinoma together with *BRCA2* and other two genes in this region. The occurrence of carcinoma in this family is 70-100% for two generations, much higher than *BRCA2* mutation alone, suggesting that an additional gene in this region is involved in cancer development. I have clone the human Tao3p C-terminus into the two-hybrid system, found that like its yeast homologue, human Tao3p C-terminus part also interacted with Cdc13p, but not Cdc13-1p. These data suggested that the function of Tao3p may be conserved.

d. two-hybrid interactions between Cdc13p and Hdf1p, Hdf2p; Pol1p and Hdf1p, Hdf2p

Hdf1p and Hdf2p are the two subunit of the Ku complex in yeast(Boulton and Jackson, 1996. Null mutant of hdf1 or hdf2 is viable at 30OC, but senescence quickly at 37OC; Boulton and Jackson, 1998). Telomeres are shorter than wild type, and there are constitutive single-stranded G-tails at telomeres(Gravel et al., 1998). The constitutive G-tails could be due to in C-strand synthesis by Pol α or due to a failure in C-strand protection. I tested the two-hybrid interactions between Cdc13p and Hdf1p, Hdf2p, Pol1p and Hdf1p, Hdf2p. The results were all negative. I also made the double mutation *hdf1::HIS3 pol1-236,* reasoning that if Hdf1p is in the same pathway as Cdc13p in terms of recruiting Pol α activity, the double mutants would have a more severe phenotype. They might senesce faster and have shorter telomeres. These results were also negative. The double mutation senesced slower and had slightly longer telomeres than *hdf1* null. These data suggest that Hdf1p, unlike Cdc13p, is not involved in C-strand synthesis. It is more likely involved in C-strand protection(midterm report).

e. Two-hybrid interaction between Cdc13p and Stn1p, Pol1p and Stn1p

Telomere protein Stn1p is a Cdc13p interacting protein(Grandin et al., 1997). The *stn1-13* mutant has a similar phenotype as the *cdc13-1*. These two proteins might potentially form a complex *in vivo*. Therefore, Stn1p might be also involved in recruiting Pol α activity. To test this possibility, full-length *STN1* was cloned into the prey vector in the two-hybrid system. Consistence with C. Nugent's data (Personal communication), it interacts with Cdc13p and Cdc13-1p, but not with Cdc13-2p. Stn1p also interacts with the N-terminal fragment of Pol1p in the two-hybrid assay. I will test if the interaction is

independent of Cdc13p and if the interaction is a direct physical interaction(midterm report).

f. Cdc13-yeast PCNA interaction

Pol30p is the PCNA homologue in budding yeast. It is the processive factor in conventional DNA replication. It might also play an important role in DNA repair, cell cycle checkpoint by interacting with proteins involved in DNA repair and check point. It forms a trimer ring structure, capable of encircling and sliding on the double stranded DNA at the replication forks during DNA replication.

It is very exciting that this protein interacts with Cdc13p in a two-hybrid assay. Amino acid residues involved in interaction with Cdc13p have been mapped by a PCR mediated random mutagenesis. They all located at the inner surface phase which encircles DNA. Single mutations in Cdc13p that disrupt the interaction were also isolated using the random mutagenesis. This interaction was found to be independent of Cdc13-Pol1p interaction, suggesting a possible direct interaction between Cdc13p and Pol30p.

Interestingly, Cdc13-1p, the mutated protein that leads to a unlimited C-strand degradation in cells, does not interact with Pol30p. In addition, at least one additional mutation in Cdc13p, Cdc13-510p, that lost the interaction showed the same phenotype as Cdc13-1p: accumulating ss-G-tails at restrictive high temperatures. Based on these data, I propose that the Cdc13p-Pol30p interaction is important for limiting C-strand degradation.

g. *pfh1* is an essential *S. pombe* helicase.

Pif1p functions at inhibition of telomerase activity through its DNA helicase activity in *S. cerevisiae*(Schulz, V. P. and Zakian, 1994, Zhou J. Q. et. al., 2000). However Pif1p is not essential. *Pfh1* is a homologue of *PIF1* in *S. pombe*. We found that the *pfh1* is an essential gene, and its helicase activity is responsible for its essential function. (Details are in the manuscript submitted to MCB)

Key Research Accomplishments

-Identified an interaction between Cdc13p and Pol1p, the catalytic subunit of DNA polymerase α.

-Identified an interaction between Cdc13p and Est1p, an essential protein for telomerase activity.

-Showed that Cdc13p and Pol1p are in the same complex in vivo by immunoprecipitation.

-Showed that yeast cells develop abnormally long telomeres with no noticeable growth defect if they express any one of the mutant *pol1* proteins or *cdc13* mutant proteins.

-Concluded that the Pol1p subunit of DNA polymerase α is responsible for C-strand re-synthesis during telomere replication.

-Suggested that telomere length is regulated by the interactions of Cdc13p with Pol1p and Est1p to balance opposite directions of G-strand and C-strand synthesis.

-Proposed models for regulation: Competition or Coordination.

-Proposed model for Cdc13p recruiting telomerase activity: Cdc13p-Est1p interaction results a conformational change that favors the recruitment of telomerase activity.

-Discovered two-hybrid interactions between Cdc13p and Fun12p, and Cdc13p and Tao3p.

-Isolated single-amino-acid mutations in Fun12p that disrupted the interaction with Cdc13p.

-Showed that the deletion of Tao3p or the Cdc13p interacting domain in Tao3p gives the same phenotype as *tao3* null cells: multiple budding, cell aggregation and modest shorter telomeres .

-Discovered that Hdf1p is not involved in C-strand synthesis. Hypothesized that Hdf1p is involved in C-strand protection.

-Discovered a two-hybrid interaction between Pol1p and Stn1p.

-Discovered a yeast two-hybrid interaction between Cdc13p and yeast PCNA

-Isolated a temperature sensitive mutation in Cdc13p that lost interaction with yeast PCNA, in addition to cdc13-1

-These two ts mutants in cdc13p have a common feature: accumulation of G-tails due to unlimited C-strand degradation

-Proposed that the Cdc13p-yPCNA might be important for the C-strand regulation.

-Discovered that the pfh1 is a helicase and its helicase activity is essential.

Reportable Outcomes

Publication:

Haiyan Qi and Virginia A. Zakian. "The *Saccharomyces* telomere binding protein Cdc13p interacts with both the catalytic subunit of DNA polymerase α and the telomerase associated Est1 protein" *Gene & Develop.* (2000) **14**(14): 1777-1788.

Manuscript

Jin-Qiu Zhou*, **Haiyan Qi***, Vincent P. Schulz, Maria K Mateyak, Ellen K. Monson and Virginia A. Zakian. *S. pombe pfh1*$^+$ encodes an essential 5' to 3' DNA helicase that is a member of *PIF1* sub-family of DNA helicase. *MCB*. Submitted. *co-first authors.

Abstract

Haiyan Qi . Yeast PCNA interacts with Cdc13p. Eukaryotic DNA replication. 2001 FASEB Summer Conference. Page 187

Products:

Created yeast strains that express mutant forms of Pol1p, Cdc13p, Fun12p, yeast PCNA, Tao3p and *pfh1*.

Conclusions:

Cdc13p plays an important regulatory role in yeast telomere replication: limits C-strand degradation, recruits telomerase and DNA polymerase α activity, and regulates G-strand and C-strand synthesis in telomere replication.

Pol α is the C-strand synthesis enzyme.

Ku complex is not involved in C-strand re-synthesis, but might be involved in C-strand protection.

Cdc13p and yeast PCNA interaction might be important for limiting C-strand degradation.

Tao3p has multiple roles in cell growth: coordinated budding and DNA synthesis, mother-daughter cell wall separation and possible telomere function.

S. pombe pfh1$^+$ encodes an essential 5' to 3' DNA helicase that is a member of *PIF1* sub-family of DNA helicase.

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Zhou J.-Q. *, Qi, H._*, Schulz, V. P., Mateyak, M. K., Monson, E. K. and Zakian V. A. *S. pombe pfh1*$^{+}$ encodes an essential 5' to 3' DNA helicase that is a member of *PIF1* sub-family of DNA helicase. *MCB*. Submitted. *co-first authors.

Schizosaccharomyces pombe pfh1^{+} encodes an essential 5' to 3' DNA helicase that is a member of the *PIF1* sub-family of DNA helicases

Running title: *S. pombe* Pfh1p helicase

Key words: fission yeast, telomere, DNA replication

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ABSTRACT

The *Saccharomyces cerevisiae* Pif1p DNA helicase is the prototype member of a helicase sub-family conserved from yeast to humans. *S. cerevisiae* has two *PIF1*-like genes, *PIF1* itself and *RRM3* that have roles in maintenance of telomeric, ribosomal, and mitochondrial DNA. Here we describe the isolation and characterization of *pfh1*$^+$, a *Schizosaccharomyces pombe* gene that encodes a Pif1-like protein. Pfh1p was the only *S. pombe* protein with high identity to *Saccharomyces* Pif1p. Unlike the two *S. cerevisiae* Pif1 sub-family proteins, the *S. pombe* Pfh1p was essential. Like *Saccharomyces* Pif1p, a truncated form of the *S. pombe* protein had 5' to 3' DNA helicase activity. Point mutations in an invariant lysine residue in the ATP binding pocket of Pfh1p had the same phenotype as deleting *pfh1*$^+$, demonstrating that the ATPase/helicase activity of Pfh1p was essential. Mutant spores depleted for Pfh1p proceeded through S phase and arrested with a terminal phenotype consistent with a defect in DNA replication. Telomeric DNA was modestly shortened in the absence of Pfh1p. However, genetic analysis demonstrated that maintenance of telomeric DNA was not the sole essential function of *S. pombe* Pfh1p.

INTRODUCTION

The *Saccharomyces cerevisiae PIF1* is a non-essential gene that encodes a 5' to 3' DNA helicase (Lahaye et al., 1991) that was first identified because it is required for the stable maintenance of mitochondrial DNA (Foury and Kolodynski, 1983). In addition, there is a nuclear form of Pif1p that affects telomeres (Schulz and Zakian, 1994; Zhou et al., 2000). Telomere length is inversely proportional to the amount of Pif1p: cells lacking Pif1p have long telomeres whereas cells over-expressing Pif1p have short telomeres. The catalytic activity of Pif1p is required for its effects on telomeres as point mutations that eliminate the helicase activity of Pif1p have the same phenotypes as null alleles (Zhou et al., 2000). Pif1p also inhibits telomerase mediated addition of telomeric DNA to spontaneous and induced double strand breaks (Mangahas et al., 2001; Myung et al., 2001; Schulz and Zakian, 1994; Zhou et al., 2000). Because Pif1p is associated with telomeric DNA *in vivo*, its effects on telomeres are likely direct (Zhou et al., 2000).

The *S. cerevisiae PIF1* is the founding member of a helicase subfamily with homologues found in other fungi, *C. elegans*, *D. melanogaster*, and *H. sapiens* (Bessler et al., 2001; Zhou et al., 2000). *S. cerevisiae* has a second *PIF1*-like gene, *RRM3* that encodes a protein that is 38% identical to Pif1p over a 485 amino acid region. Like *PIF1*, *RRM3* is not essential; moreover, cells lacking both genes are also viable (Ivessa et al., 2000). *RRM3* was first identified because its mutation increases recombination in the ribosomal DNA (rDNA) (Keil and McWilliams, 1993). However, the effects of Rrm3p on rDNA recombination are probably a secondary consequence of defects in rDNA replication (Ivessa et al., 2000). In the absence of Rrm3p, replication stalls at multiple sites within the rDNA. Separation of converging forks is especially impaired in *rrm3* cells. The effects of Rrm3p on rDNA replication require its catalytic activity

and are probably direct, as Rrm3p is rDNA associated *in vivo* (Ivessa et al., 2000). Rrm3p also affects fork progression within sub-telomeric and telomeric DNA. As in rDNA, sister chromatid separation within telomeric regions is delayed in a *rrm3* strain (A.S. Ivessa, J.-Q. Z, V.P.S., E.K.M. and V.A.Z., submitted).

Most helicases contain seven short motifs spread throughout a 300-500 amino acid region (Ellis, 1997). Because these motifs are short and degenerate, their presence alone is not sufficient to confer significant sequence similarity upon proteins containing them. For example, *S. cerevisiae* has 134 open reading frames with helicase-like features (Shiratori et al., 1999), yet Rrm3p is the only *S. cerevisiae* protein with significant similarity to Pif1p by the criterion of a TBLASTN search (Bessler et al., 2001; Zhou et al., 2000). Helicase subfamilies, such as the Pif1 subfamily, are defined as a group of proteins that have highly significant sequence similarity to each other but not to other helicases. The functional significance of the sequence similarity that defines helicase subfamilies is not known. The sequence similarity might reflect common functions, recognition of a common nucleic acid substrate, or interaction with a common co-factor.

As part of our goal to understand the functions of the *PIF1* subfamily of DNA helicases, we isolated and characterized a *PIF1*-like gene from the fission yeast *Schizosaccharomyces. pombe*, called *pfh1*$^+$ (***PIF1*** **h**omologue). *S. pombe* and *S. cerevisiae* are distantly related, about as similar to each other as each is to humans (Russell and Nurse, 1986), yet many genes involved in telomere replication and chromosome maintenance are conserved between them. Although the three *S. pombe* chromosomes are each much larger than any of the sixteen *S. cerevisiae* chromosomes, other features of genome organization are similar. For example, in both organisms telomeres are ~300 bps in length and have an irregular sequence, $C_{1\text{-}3}A/TG_{1\text{-}3}$ in *S. cerevisiae* (Shampay et al., 1984) and $C_{2\text{-}8}A_2TCT/\ T_2G_{2\text{-}8}ACA$ in *S. pombe* (Hiraoka et al., 1998;

Sugawara, 1989). Likewise, ribosomal DNA is organized and replicated similarly in both organisms (Brewer and Fangman, 1988; Linskens and Huberman, 1988; Sanchez et al., 1998). In contrast, the *S. cerevisiae* mitochondrial DNA is much larger (~75 kb; reviewed in Pon and Schatz, 1991) than in *S. pombe* (~19 kb; Lang and Wolf, 1984), and unlike wild type *S. pombe*, *S. cerevisiae* can live without mitochondrial DNA.

Our analysis of Pfh1p showed that it had roughly equivalent similarity to both Pif1p and Rrm3p, ~60% similar over a ~450 amino acid region that contains the seven helicase motifs. We purified a truncated form of Pfh1p and demonstrated that, like Pif1p (Lahaye et al., 1991), it had 5' to 3' DNA helicase activity. Unlike both of its *Saccharomyces* homologues, *pfh1*$^+$ was essential, and its catalytic activity was required for its essential function. Although cells lacking Pfh1p had telomeres that were ~ 50 bps shorter than wild type cells, maintenance of telomeric DNA was not the sole essential function of Pfh1p.

MATERIALS AND METHODS

General Methods and Nomenclature. All *S. pombe* strains were isogenic to wild type strain 972 h$^-$. Strains constructed by integrative transformation (Rothstein, 1983) were confirmed by Southern blotting. Adhering to the conventions appropriate for each yeast species, the wildtype and mutant versions of the Saccharomyces *PIF1* gene were designated, respectively, *PIF1* and *pif1* whereas the wild type and mutant versions of the *S. pombe PIF1*-like gene were designated, respectively, *pfh1*$^+$ and *pfh1*$^-$. Deletion alleles are noted by a delta symbol in *S. cerevisiae* and by a D in *S. pombe* .

Identification and analysis of *pfh1*$^+$. The *S. pombe pfh1*$^+$, was identified using nested degenerate PCR. *S. pombe* genomic DNA was first amplified using the FIE/DMNL primer TCGAATTCT(C/T)AT(A/C/T)GIATG(C/T)TIA and the QAYVAL primer

CAAAGCTTA(A/G)IGCIAC(A/G)TAIGC(CT)TG, where I stands for inosine. Reaction products of 500 to 1300 base pairs were isolated and re-amplified using the GAQVM primer TCGAATTCGGIGCICA(A/G)GTIATG and the QGQTL primer CAAAGCTTA(A/G)IGT(C/T)TGICC(C/T)TG. The major PCR product of 450 bp was isolated, cloned, and sequenced. To obtain the rest of the *pfh1*$^+$ gene, the PCR restriction fragment was used as a probe to screen a *S. pombe* genomic library (generously provided by Wayne Wahls of Vanderbilt University). Nested deletions of *pfh1*$^+$ for sequencing were generated as described (Davies and Hutchinson, 1991). Sequencing was done with a combination of manual Sequenase (USB/Amersham) and automated dye terminator reactions. Both strands of the *pfh1*$^+$ gene were fully sequenced. The *pfh1*$^+$ sequence was analyzed using GCG software (Group, 1994). The sequence was deposited in the database (Accession number AF074944).

The splicing of the 41 bp intron was confirmed by sequencing a *pfh1*$^+$ cDNA. The *pfh1*$^+$ cDNA was obtained by amplifying a *S. pombe* cDNA library (Becker et al., 1991) using the primer 5' ACATTTAATAAACACAGAAG and 5' GATCCTAAAAGAACCAGCAC. The resulting PCR product was cloned, and the region of the insert that contains the intron was sequenced. The 5' end of the *pfh1*$^+$ gene was also identified by amplification of the *S. pombe* cDNA library. The library was first amplified with an ADC promoter primer 5' CTTTTTCTGCAGAATATTTCAAGCTATACC and *pfh1*$^+$ primer SP1403 5' TTAGTACATGGTAAACTCGT. This PCR product was re-amplified using the ADC primer and primer 5' TCAAAAACGGGCAGTGAGGG. The PCR product was cloned, and sequenced.

Construction and analysis of *pfh1-D1: ura4*$^+$ mutant strains: The plasmid pVS110 contains a 2.9 kb *Sau*3A genomic DNA fragment generated by partial digestion with *Sau*3A that begins 35 bp 5' of the start codon and extends to the *Cla*I site at the end of the sequence. In the deletion plasmid, the two *Eco*RV internal *pfh1*$^+$ fragments, a 1492 bp region, were replaced with a 1.8 kb

*Sph*I fragment containing *ura4*$^+$ (deleted region is indicated by triangles in Supplementary material). An insertion allele was made by inserting the *ura4*$^+$ fragment into the *Stu*I site of *pfh1*$^+$. The integration was performed using diploid strain #585 with the genotype *his3-D1/his3-D1 leu1-32*$^-$*/leu1-32*$^-$ *ura4-D18/ura4-D18 ade6-m210*$^-$*/ade6-M216*$^-$ *h*$^+$*/h*$^-$ by selecting for Ura$^+$ transformants. This diploid strain was made by mating strains of genotype *his3-D1 leu1-32*$^-$ *ura4-D18 ade6-m210*$^-$ h$^+$ and *his3-D1 leu1-32*$^-$ *ura4-D18 ade6-M216*$^-$ h$^-$ which were kindly provided by Kathy Gould (unpublished strains).

For sporulation experiments, overnight cultures of diploid strains #584 (*pfh1-D1*::*ura4*$^+$ */pfh1*$^+$ *his3-D1/ his3-D leu1-32/ leu1-32 ura4-D18/ura4-D18 h*$^+$*/h*$^-$ *ade6-M210/ade6-M216)* and #585 (*pfh1*$^+$*/pfh1*$^+$ *his3-D1/his3-D1 leu1-32/ leu1-32 ura4-D18/ura4-D18 h*$^+$*/h*$^-$ *ade6-M210/ade6-M216)* were grown in YEA medium. For sporulation, 100 microliters of each culture were spread onto YEPD medium for two days at 30°. The plates were scraped and cells resuspended in water to a final cell density of ~2 - 5 x 10^7 cells/ml. Glusulase (Dupont) was added to 0.5% and cells were incubated overnight at 30° and then spores were harvested. For germination, 10^8 spores were innoculated into 50 ml EMM medium that contained sodium glutamate (EMMG) instead of ammonium chloride as the nitrogen source. Spores derived from the *pfh1-1D*::*ura4*$^+$ */pfh1*$^+$ heterozygote #584 were inoculated into EMMG without uracil. In this medium, only spores that had the *pfh1-D1*::*ura4*$^+$ deletion were able to germinate and grow. Wild-type spores derived from the *pfh1*$^+$*/ pfh1*$^+$ homozygous control were inoculated into EMMG with uracil. Samples of the germinating spores were taken at various time points, harvested, fixed in 70% ethanol, digested with RNase A, and either stained with Yo-Yo 2 (Molecular Probes) for confocal microscopy or with propidium iodide for fluorescence activated cell sorting (FACS) analysis (Alfa et al., 1993).

For Southern blot analysis, DNA was prepared by a glass bead procedure (Runge and Zakian, 1989), digested with *Hin*fI or *Apa*I, and run on a 1% agarose gel. The blots were hybridized sequentially with a telomeric probe (from plasmid pSPT16; (Sugawara, 1989), a probe for the entire *S. pombe* mitochondrial genome (from plasmid pDG3; (Del Giudice, 1981), a 288 bp probe that detects the *rhp6*$^+$ gene that was made by PCR amplification of genomic DNA (Reynolds et al., 1990), and to a 2.4 kb rDNA probe made by PCR amplification. Probes were labeled with α^{32}P-dCTP using the RTS RadPrime DNA labeling system (Gibco BRL).

Complementation analysis of the *pfh1-D1*::*ura4*$^+$ deletion strain was performed by plasmid swapping. A 6.1 kb *S. pombe* genomic DNA fragment was inserted into Bluescript KSII vector. The 6.1 kb insert began 1.7 kb upstream of the first AUG of *pfh1*$^+$ and ended 1.9 kb after the *rph*$^+$ stop codon. The 6.1 kb fragment was released from the Bluescript KSII vector by digestion with *Not*I and *Pst*I, treated with T4 DNA polymerase to generate blunt ends, and inserted into the *Sma*I site of pBG2 (Burke and Gould, 1994), a plasmid containing the *S. pombe* *his3*$^+$ gene and *ars1*. This plasmid, called pVS117, was transformed into a heterozygous *pfh1-D1*::*ura4*$^+$/*pfh1*$^+$ diploid strain, and the diploid was sporulated to yield a haploid with genotype *pfh1-D1*::*ura4*$^+$ *his3-D1 leu1-32*$^-$ *ura4-D18 ade6-m210*$^-$ *or ade6-M216*$^-$ *h*$^-$/pVS117. The haploid strain was transformed with either of two plasmids, pREP41X or pREP81X, that carry the *pfh1*$^+$ gene under the control of the thiamine regulated *nmt* promoters (Basi et al., 1993).

The viability of a *ptp1-1*$^-$ *pfh1-D1*::*ura4*$^+$ double mutant was tested by mating *S. pombe* strain PTP25 (*h*$^+$ *ura1-161*$^-$ *ade6-216*$^-$ *ptp1-1*$^-$ *rho*$^+$) obtained from T. Fox (Haffter and Fox, 1992) with a strain of genotype *h*$^-$ *his3-D1 leu1-32*$^-$ *ura4-D18 ade6-M210*$^-$ *pfh1-D1*::*ura4*$^+$/pVS117. Single amino acid changes (K337A and K337R) in Pfh1p were made using the Quick-Change™ Site Directed Mutagenesis Kit (Stratagene) and VPS603 plasmid, a *his3*$^+$

plasmid containing *pfh1*$^+$. To make K337A, oligonucleotides K337A5' and K337A3' were used (K337A5': 5'-gct gga aca ggC GCC tct gtt ctc ct-3'; K337A3': 5'-ag gag aac aga GGC Gcc tgt tcc agc-3'). (Mutated residues are capitalized.) This pair of oligonucleotides generated a *Sfo*I site near K337 in *pfh1* without changing any additional amino acid, which facilitated identification of the K337A mutation. To make K337R, oligonucleotides K337R5' and K337R3' were used (K337R5': 5'-ct gga aca ggt CGa tcG gtt ctc ctc cg-3', K337R3': 5'-cg gag gag aac Cga tCG acc tgt tcc ag-3'.) This pair of oligonucleotides generates a *Pvu*I site near K337 without changing any additional amino acids.

To determine if *pfh1*$^+$ was essential in cells that lack telomeric DNA, we used strains CF199 (*h- leu1-32 his3-D1 ura4-D18 ade6-M210*) (the wild type control) and CF448 (isogenic to CF199 except for *trt1*::*his3*$^+$ and having circularized chromosomes), both kindly supplied by T. Nakamura (Nakamura et al., 1998). Strains were transformed with a 2.9 kb *Eco*RI/*Pst*I/*Bgl*I fragment from pVS110 which contained the *pfh1*::*ura4*$^+$ deletion/disruption cassette with or without the *LEU2* plasmid pREP41X-*pfh1*$^+$ (the *S. cerevisiae LEU2* gene complements a *S. pombe leu1-32* strain.)

Purification and analysis of Pfh1p-h

To express active recombinant Pfh1p, a 1608 bp fragment that encoded amino acids 255 to 789 of Pfh1p, was isolated from the *pfh1*$^+$ cDNA, cloned into the *Sal*I site of pEG(KT) (Mitchell et al., 1993) to generate pEG(KT)-Pfh1-h, and transformed into a protease-deficient *S. cerevisiae* strain BCY123 (Bennett et al., 1998). Expression of the GST-Pfh1p-h fusion protein was carried out using minor modifications of methods described in (Bennett et al., 1998). Purification was carried out at 4° C. Cells were harvested, washed and resuspended in 8-volume of ice-cold lysis buffer (50 mM TrisHCl, pH 7.8, 500 mM NaCl, 4 mM MgCl2, 40 μg/ml DNase I, 10 mM dithiothreitol, 0.1% Triton X-100, 0.004% 1-octonal) and a mixture of protease

inhibitors (10 μg/ml aprotinin, 5 μg/ml leupeptin, 10 μg/ml pepstatin A, 100 μg/ml bacitracin, 250 μg/ml soybean trypsin inhibitor, 0.4 mM phenylmethylsulfonyl fluoride and 10 mM benzamidine hydrochloride). Cells were lysed by two passes through a cell disruptor (EmulsiFlex-C5, AVESTIN). After centrifugation (15,000 g, 15 min), the supernatant was brought to 50% saturation with ammonium sulfate and left on ice for 30 min. The precipitate was collected by centrifugation (27,000 g, 30 min), suspended in 20 ml PBS supplemented with 5 mM dithiothreitol, 0.5% Triton X-100, 0.001% 1-octonal, and protease inhibitor mix. The soluble fraction was recovered by centrifugation (27,000g for 30 min) and loaded onto a 4 ml glutathione sepharose 4B column (1.6 x 2) equilibrated with PBS, at a flow rate of 20 ml/hr. The column was washed with 20-bed volumes of the same buffer and 20-bed volume of the same buffer containing 1 M NaCl. Protein was eluted with 10 ml of elution buffer (50 mM TrisHCl, pH 8.8, 30 mM reduced glutathione, 50 mM NaCl, 10 mM dithiothreitol, 0.1% triton X-100, 0.001% 1-octanol). The eluate was digested with 200 units of thrombin overnight, and loaded onto a 1 ml heparin column equilibrated with 50 mM TrisHCl buffer, pH 7.8 (200 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.002% triton X-100) at a flow rate of 30 ml/hr. The column was washed with the equilibration buffer, and eluted with linear NaCl gradient from 200 mM to 1 M in the same buffer and 1ml fractions were collected. Recombinant Pfh1p-h eluted between 700 and 800 mM NaCl, as determined by Commassie Blue stained SDS-PAGE and Immunoblot analysis. The peak fractions were pooled and concentrated by Centricon (Millipore) centrifugation. The Pfh1p antibodies used to follow expression and purification of Pfh1p-h was made against amino acids 447 to 708 of Pfh1p expressed in *E. coli* as a GST-fusion protein. The fusion protein was purified according to Pharmacia Biotech protocols, and used both to raise polyclonal serum in rabbits and to make an affinity column for the purification of the antiserum as described in (Koff et al., 1992).

ATPase reactions were carried out in 20 µl ATPase buffer (25 mM Hepes, pH 7.6, 5 mM MgCl2, 2 mM ATP, 1 mM dithiothreitol, 100 µg/ml of BSA, 200 µg/ml M13 single strand DNA, with 200 ng of recombinant Pfh1p-h or 100 ng of recombinant Pif1p), for 30 min at 37°C. Each reaction contained 0.5 µCi of [γ-^{32}P] ATP. Reactions were stopped by the addition of 1 µl of 0.5 M EDTA, and 0.5 µl of each reaction was spotted on polyethylimine (PEI) cellulose plate (Baker). The plate was developed in 0.8 M LiCl, and dried with hot air. The ATP hydrolysis was visualized on a Molecular Dynamics Phosphoimager. For helicase assays, a 25-mer (5'-GTTGTAAAACGACGGCCAGTGAATT-3'), and 36-mer (5'-CGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATT-3') oligonucleotides were annealed to single-stranded M13mp7 or M13mp18 DNA. For the helicase assay, 10 pmol of the 36-mer was ^{32}P-labeled at its 5' end using T4 polynucleotide kinase and [γ-^{32}P]ATP, and 2.5 pmol was annealed with equal molar single-stranded M13mp18 virion DNA in a 75 µl reaction mixture. For the helicase polarity assay, 2.5 pmol each of the ^{32}P-labeled 36-mer and of the ^{32}P-labeled 25-mer were annealed with equal molar single-stranded M13mp7 DNA linearized by digestion with *Eco*RI. The labeled substrates were purified with the Chroma Spin-1000 column (Clontech). Helicase assays (20 µl each) contained 20 mM Hepes, pH 7.6, 5 mM $MgAc_2$, 4 mM ATP, 100 µg/ml BSA, 5% glycerol, 1 mM DTT, 2 µl DNA substrate, and 200 ng of recombinant Pfh1p-h protein (or 100 ng recombinant Pif1p) and carried out for 10 min at 37°C. The amount of Pfh1p-h used is comparable to that used to detect helicase activity of the *S. cerevisiae* Pif1p (Lahaye et al., 1993; Lahaye et al., 1991; Zhou et al., 2000) as well as other *S. cerevisiae* DNA helicases (Sedman et al., 2000; Sung et al., 1988) and the Mcm complex from *S. pombe* (Lee and Hurwitz, 2001) and other organisms (Chong et al., 2000; You et al., 1999). Products were

analyzed by electrophoresis in a 10% polyacrylamide (89 mM Tris borate, pH 8.3, 2 mM EDTA), and visualized on a Molecular Dynamics Phosphoimager.

RESULTS

Isolation of a *PIF1*-like gene from *Schizosaccharomyces pombe*.

We identified a *PIF1*-like gene from the fission yeast *Schizosaccharomyces pombe* using nested degenerate PCR. To obtain a clone that contained the entire *S. pombe PIF1*-like gene, hereafter called *pfh1*$^+$, for ***PIF1*** **h**omologue, the PCR restriction fragment was used as a probe to screen a *S. pombe* genomic library (Fikes et al., 1990). Both strands of the *pfh1*$^+$ gene were sequenced, and the sequence deposited in the database (Accession number AF074944). (The DNA sequence and predicted protein sequence is shown in Supplementary material).

The conceptual translation of the *pfh1*$^+$ gene generated an 805 amino acid protein with one small 41 base intron whose presence was confirmed by sequencing a cDNA obtained from a random library of *S. pombe* cDNA clones (data not shown) There were two out of frame AUG codons in the 5' end of the mRNA before the first AUG of the *pfh1*$^+$ open reading frame (ORF) (Supplementary material, labeled M and underlined). The presence of the two upstream AUG codons suggests that *pfh1*$^+$ is either translationally regulated or poorly translated, since eukaryotic translation usually initiates at the first AUG in an mRNA (Kozak, 1992). There are three *Mlu*I sites (MCB, *Mlu*I cell cycle box) 5' of the start of the *pfh1*$^+$ ORF (Supplementary material; denoted by bold letters and dotted lines). *Mlu*I sites are found in the promoter regions of many *S. cerevisiae* and *S. pombe* DNA synthesis genes (reviewed in McIntosh, 1993).

When the predicted protein sequence of *S. pombe* Pfh1p was compared to the translated DNA database using the TBLASTN program (Altschul et al., 1990), it displayed highly significant similarity to each of the previously described (Bessler et al., 2001) Pif-like proteins (Table I). Since our last analysis of *PIF1*-like genes (Bessler et al., 2001), several additional

Pif1-like proteins were reported. *Neurospora crassa* and the basidiomycete *Phanerochaete chrysosporium* both encode two Pif1p-like proteins. Like multi-cellular eukaryotes (but unlike *S. cerevisiae*, *N. crassa*, and *P. chrysosporium*), *S. pombe* encoded only a single Pif1-like protein. Although the helicase domain of Pfh1p was ≥36% identical to each of the other Pif1-like proteins (Zhou et al., 2000), its amino terminal ~330 and its carboxyl terminal 52 amino acid were not related to these portions in other Pif1 sub-family members.

The *S. pombe* Pfh1p is a 5' to 3' DNA helicase.

To determine if Pfh1p is also a DNA helicase, we purified and characterized recombinant Pfh1p. Initially we expressed full-length Pfh1p in *E. coli*, but this protein had poor solubility and was often degraded. Therefore, a truncated version of Pfh1p consisting of amino acids 255 to 789 of the 805 amino acid protein that contained all seven helicase motifs, was fused at its amino terminus to GST to generate Pfh1p-h (h stands for helicase) (the portion of Pfh1p that was expressed is indicated by brackets in the Supplementary material). DNA encoding the GST-Pfh1p-h fusion protein was introduced into *S. cerevisiae* under the control of a galactose inducible promoter. Protein extracts were prepared from both galactose and glucose grown cells and examined by Western analysis using affinity purified anti-Pfh1 antibodies (Fig. 1). The galactose grown cells (induced) contained an ~84-kDa protein, the appropriate size for the GST-Pfh1p-h fusion protein, that was not detected in control extracts (Fig. 1B, compare uninduced in lane 2, to induced by galactose addition in lane 3). The 58.8 kDa truncated Pfh1p-h was purified to near homogeneity as described in materials and methods (Fig. 1A, lane 6). The faint Coomassie blue staining bands in the preparation of purified Pfh1p-h (Fig. 1A) were detected by the anti Pfh1p antibodies (Fig. 1B), indicating that they were likely degradation products of GST-Pfh1p-h.

The GST-Pfh1p-h fusion protein had no detectable ATPase or helicase activity (data not shown). To remove the GST moiety, GST-Pfh1p-h was cleaved with thrombin and fractionated on Heparin sepharose. The purified recombinant Pfh1p-h had Mg^{2+} dependent ATPase activity (Fig. 2A) that was stimulated by single strand DNA (Fig. 2A, lane 5). Helicase activity of recombinant Pfh1p-h was demonstrated by its ability to displace a ^{32}P labeled 36-mer oligonucleotide annealed to single-stranded circular M13 DNA, a reaction that was both Mg^{2+} and ATP dependent (Fig. 2B, C). Because the ATPase and helicase activities were not detectable until the GST-Pfh1p-h was thrombin cleaved, these enzymatic activities were due to Pfh1p-h itself, rather than to a minor contaminating species.

The polarity of the recombinant Pfh1p-h helicase was established using a substrate in which kinase-labeled 25- and 36-mer oligonucleotides were annealed at, respectively, the 5' and 3' ends of linearized single-stranded M13 DNA (Fig. 2C). A 5' to 3' DNA helicase will displace the 36-mer while a 3' to 5' DNA helicase will remove the 25-mer. Like purified recombinant Pif1p (Fig. 2C, lane 6), recombinant Pfh1p-h displaced the 36-mer but not the 25-mer from the M13 DNA (Fig. 2C, lane 5). Thus, Pfh1p-h is a 5' to 3' DNA helicase.

The *S. pombe pfh1*$^+$ gene is essential.

Integrative transformation was used to delete 1492 base pairs from one of the two copies of *pfh1*$^+$ in a diploid strain. The deletion was marked by insertion of the *ura4*$^+$ gene in an otherwise *ura4*$^-$ strain. The *pfh1*$^+$/*pfh1-D1*::*ura4*$^+$ diploid was sporulated, and 100 random spores were all found to be *ura4*$^-$. When tetrads from a *pfh1*$^+$/*pfh1-D1*::*ura4*$^+$ diploid were dissected, only two of the four spores formed large colonies, and the large colonies were all *ura4*$^-$ confirming that *pfh1*$^+$ was an essential gene (Fig. 3A). The *pfh1-D1*::*ura4*$^+$ spores formed microcolonies with 1-10 cells in which the individual cells were often elongated (as in Fig. 3B).

We constructed versions of *pfh1*$^+$ that were expressed from the thiamine repressible *nmt* promoter (Basi et al., 1993; Maundrell, 1993). These plasmids were introduced into the *pfh1*$^+$/*pfh1-D1*::*ura4*$^+$ diploid strain and the diploid sporulated. Even when the weakest *nmt1* promoter was used, the *nmt1*-driven *pfh1*$^+$ gene was able to complement the *pfh1-D1* strain in medium containing thiamine (data not shown). This *nmt*-driven *pfh1*$^+$ gene's ability to rescue the lethality of the *pfh1-D1*::*ura4*$^+$ strain showed that the phenotypes attributed to loss of Pfh1p were not due to reduced expression of a neighboring gene and also demonstrated that very little Pfh1p was needed for viability.

S. pombe cells lacking Pfh1p proceed through S phase but have shorter telomeres and an elongated cellular phenotype.

To obtain larger numbers of *pfh1-D1*::*ura4*$^+$ cells, the *pfh1*$^+$/*pfh1-D1*::*ura4*$^+$ diploid was sporulated and then spores were grown in liquid medium lacking uracil. As a control, a *pfh1*$^+$/ *pfh1*$^+$ *ura4*$^-$ / *ura4*$^-$ diploid strain was treated in the same way. As demonstrated by others, *ura4*$^-$ spores do not germinate in medium lacking uracil (see, for example, Waseem et al., 1992). As expected, no germinated spores were seen in the wild type culture. In contrast, the *pfh1*$^+$/*pfh1-D1*::*ura*4$^+$ culture germinated and at least some cells divided one or more times, producing a culture in which cell number was two to three fold higher than in the starting culture. Since only the *pfh1-D1*::*ura4*$^+$ cells could divide in the absence of uracil, these methods yielded a population of cells depleted for Pfh1p.

The DNA of the *pfh1-D1*::*ura4*$^+$ cells was stained, and cells were observed by confocal microscopy (Fig. 3B). For comparison, we sporulated a *pfh1*$^+$/ *pfh1*$^+$ but otherwise isogenic diploid strain in media containing uracil (Fig. 3C). By 20 hours post sporulation, *pfh1-D1*::*ura4*$^+$ cells appeared elongated compared to wild type cells, indicative of cells that are able to grow but not divide (see, for example, Kelly et al., 1993; Waseem et al., 1992). This difference in cellular

morphology was apparent at ~14 hours post sporulation. At various times during germination, samples of wild type and the *pfh1-D1*::*ura4*$^+$ mutant cells were stained with propidium iodide and analyzed by fluorescence activated cell sorting (FACs). Like wild type cells, DNA replication was detectable 10 hours post-germination in the *pfh1-D1*::*ura4*$^+$ cells (Fig. 4). By 20-22 hours, the vast majority of cells in both the wild type and mutant cultures had a 2C DNA content. Thus, upon depletion of Pfh1p, arrested cells accumulated with replicated genomes resembling the steady-state distribution of wild type cultures.

We also examined telomere length in DNA from *pfh1-D1*::*ura4*$^+$ mutant cells (Fig. 5A). *S. pombe* has six telomeres, each bearing ~300 bp of telomeric repeats (Sugawara, 1989). Five of the six telomeres also have at least 19 kb of sub-telomeric DNA. The other telomere, which generates a 0.9 kb fragment after *Hinf*I digestion (Fig. 5A, right hand lanes) is immediately abutted to the rDNA locus (Sugawara, 1989). Telomeric DNA from *pfh1-D1*::*ura4*$^+$ cells was 40-60 bps shorter than telomeric DNA from *pfh1*$^+$ cells (Fig. 5A). The telomere adjacent to the rDNA that generates the 0.9 kb *Hinf*I fragment appeared to be less affected than the other telomeres. Although the effect on telomere length was small, it was reproducible, being seen in DNA prepared from two independent sporulations, restricted with three different enzymes (*Hinf*I and *Apa*I, Fig. 5A, and *Eco*RI, data not shown), and run on multiple gels. Similar amounts of telomere shortening were seen 16, 20 and 24 hours after germination (Fig. 5A). The shortening of DNA was specific to telomeres, since rehybridization of the same gels with a sub-telomeric (data not shown) or other internal sequences (Fig. 5B, C) showed that the mobility of these sequences was not affected. There was no detectable change in rDNA (Fig. 5B) or mitochondrial DNA (Fig. 5D) in *pfh1-D1*::*ura4*$^+$ cells.

Maintenance of telomeric DNA is not the sole essential function of Pfh1p.

Although most *S. pombe* cells die when they lack telomerase, a small subset of cells survives. In most of these survivors, the three *S. pombe* chromosomes lose the simple repeats at the very ends of the chromosomes, as well as much of the sub-telomeric TAS elements, and fuse end to end to generate circular chromosomes (Naito et al., 1998; Nakamura et al., 1998). If replication of telomeric DNA were the sole essential function of Pfh1p, it should be possible to delete *pfh1*$^+$ in a strain with circular chromosomes.

Because cells with circular chromosomes are not viable in meiosis (Naito et al., 1998; Nakamura et al., 1998), we could not sporulate a *pfh1*$^+$/*pfh1-D*::*ura4*$^+$ diploid having circular chromosomes to obtain the desired strain. Rather, we used two alternative approaches. In the first experiment, we used a *trt1*$^-$ survivor strain CF448 (Nakamura et al., 1998) that had been shown previously to have circularized chromosomes. We transformed this haploid strain with the *pfh1D1*::*ura4*$^+$ fragment that was used previously to disrupt *pfh1*$^+$ in diploid cells, selecting for Ura$^+$ cells. As a control for transformation efficiency, we transformed the same strain with both the *pfh1D1*::*ura4*$^+$ fragment and a *LEU2 pfh1*$^+$ plasmid, selecting for Ura$^+$ Leu$^+$ cells. Although we got hundreds of transformants when we co-transformed with the *pfh1*$^+$ plasmid, in two independent experiments, we obtained only a few Ura$^+$ transformants with the *pfh1* disruption fragment alone, and Southern analysis revealed that none of these rare Ura$^+$ transformants had integrated at the *pfh1*$^+$ locus. In the second approach, we disrupted the *trt1*$^+$ gene as described (Nakamura et al., 1998) in a *pfh1-D1*::*ura4*$^+$ haploid yeast that carried a *LEU2* plasmid with the *pfh1*$^+$ gene. We isolated survivors and then asked if these survivors could lose the *pfh1*$^+$ *LEU2* plasmid. Out of 700 survivors, none generated subclones that were able to grow on plates lacking leucine. In contrast, 17% of the survivors generated in a *trt1- pfh1*$^+$ strain lost a *LEU2 pfh1*$^+$ plasmid. Thus, *pfh1*$^+$ is essential even in strains that lack telomeric DNA.

The inviability of *pfh1-D1*::*ura4*$^+$ cells was not due to lack of mitochondrial DNA.

Mitochondrial DNA is lost at a high rate in *S. cerevisiae* cells that lack Pif1p (Foury and Kolodynski, 1983; Schulz and Zakian, 1994). Since wild type *S. pombe* can not survive without mitochondrial DNA (Munz et al., 1989), if Pfh1p were needed to maintain mitochondrial DNA, it would explain the inviability of *pfh1-D1*::*ura4*$^+$ strains. Although wild type *S. pombe* cells require mitochondrial DNA, cells lacking mitochondrial DNA are viable in a *ptp1-1*$^-$ strain (Haffter and Fox, 1992). Thus, if the only essential function of Pfh1p is to maintain mitochondrial DNA, a *ptp1-1*$^-$ *pfh1-D1*::*ura4*$^+$ strain should be viable. To test this possibility, a *ptp1-1*$^-$ haploid strain was mated to a *pfh1-D1*::*ura4*$^+$ strain that carried the *pfh1*$^+$ gene on a plasmid. Twenty five of the 33 tetrads that were dissected from this cross had only two viable spores, and the two non-viable spores often gave rise to cells with an elongated phenotype similar to that of the *pfh1-D1*::*ura4*$^+$ cells shown in Fig. 3B. The four tetrads with more than two viable spores were due to the *pfh1*$^+$ plasmid being retained in a *pfh1-D1*::*ura4*$^+$ spore. As 14 of 30 viable spore progeny had the *ptp1-1*$^-$ mutation, *ptp1-1*$^-$ was not genetically linked to *pfh1-D1*::*ura4*$^+$. Since the *ptp1-1*$^-$ mutation did not rescue a *pfh1-D1*::*ura4*$^+$ strain, maintenance of mitochondrial DNA can not be the sole essential function of *pfh1*$^+$. The presence of mitochondrial DNA in *pfh1-D1*::*ura4*$^+$ cells supports this interpretation (Fig. 5D).

Although *S. cerevisiae rrm3*Δ strains have no evident mitochondrial defect, deleting *RRM3* in a *pif1*Δ strain partially suppresses the loss of mitochondrial DNA caused by deleting *PIF1*. These results suggest that Rrm3p has some role in mitochondrial DNA metabolism, at least in the absence of Pif1p (VPS and VAZ, unpublished results). Although Pfh1p did not appear to be required to maintain mitochondrial DNA, it is possible that Pfh1p, like the *S. cerevisiae* Rrm3p, has some role in mitochondria. To assess if Pfh1p might function in mitochondria, we used computer programs to predict its likely sub-cellular localization. The subcellular localization program PSORT II (http://psort.nibb.ac.jp) predicted that the *S. pombe*

Pfh1p, as well as the *S. cerevisiae* Pif1p and Rrm3p, are targeted to both the nucleus and to mitochondria. In addition, we scanned Pfh1p for mitochondria targeting signals using MitoProt II (http://www.mips.biochem.mpg.de/proj/medgen/mitop/). Both PSORT II and MitoProt II base the likelihood of mitochondria targeting on the amino acid composition of the N-terminal region of the protein. MitoProt II predicts the probability of localization to the mitochondria to be 0.9877, 0.9326, and 0.9264 for Pif1p, Rrm3p, and Pfh1p respectively. Importantly, other helicases not believed to be found in the mitochondria such as Sgs1p and Rqh1p, the *S. cerevisiae* and *S. pombe* homologs of the Bloom's and Werner's syndrome helicases, were not predicted to possess mitochondria targeting sequences by these programs.

The ATPase/helicase activity is required for its essential *in vivo* function.

Some genes that encode DNA helicases, for example the *S. cerevisiae RAD3* and *DNA2* genes are essential for viability yet the helicase activity of their products is not required for their essential function (Budd et al., 2000; Formosa and Nittis, 1999; Sung et al., 1988). To determine if the helicase activity of Pfh1p is essential, we used site directed mutagenesis to modify an invariant lysine in the ATP-binding domain of motif I to either alanine (K337A) or arginine (K337R) (See supplementary material for position of this residue). Mutations in this residue eliminate the activity of all helicases in which they have been tested (Gorbalenya and Koonin, 1993), including Pif1p (Zhou et al., 2000). A *his3*$^+$ plasmid carrying either the wild type *pfh1*$^+$ gene or one of the two point mutation alleles, *pfh1*-K337A or *pfh1*-K337R, were introduced into two different *pfh1-D1*::*ura4*$^+$ strains, VPS612 and VPS613. The two *S. pombe* strains were identical except that VPS612 had a *LEU2* plasmid containing the wild type *pfh1*$^+$ gene under the control of its own promoter and VPS613 had a *LEU2* plasmid containing the *pfh1*$^+$ gene under the control of the *nmt1* promoter (the *S. cerevisiae LEU2* gene complements an *S. pombe leu1*$^-$ strain). Leu$^+$ His$^+$ transformants were isolated and then streaked two times on medium lacking

histidine but containing leucine. Growth on medium containing leucine allowed cells to lose the *LEU2* plasmid. However, Leu$^-$ cells could only be generated if the *his3*$^+$ plasmid could supply the essential function of Pfh1p. *S. pombe* strains VPS612 or VPS613 carrying the wild type *pfh1*$^+$ *his3*$^+$ plasmid readily lost the *LEU2* plasmid: in these strains, 74% or 98% of the cells were Leu$^-$ after cells were streaked twice on medium containing leucine. In contrast, Leu$^-$ cells were not recovered when the *his3*$^+$ plasmid had either the *pfh1*-K337R or *pfh1*-K337A alleles (Table II). Thus, the K337A and the K337R alleles were unable to supply the essential function(s) of Pfh1p.

DISCUSSION

The *S. pombe pfh1*$^+$ gene encodes an 805 amino acid protein with very high similarity to all other members of the Pif1p sub-family of DNA helicases (Table I). Although Pfh1p had the seven motifs that characterize helicases (see Supplementary material), it had no significant similarity by the criterion of a TBLASTN search to other *S. pombe* helicases. *S. cerevisiae* has two *PIF1*-like genes, *PIF1* itself and *RRM3*. Neither of the *S. cerevisiae* genes is essential, and *pif1 rrm3* cells are also viable (Ivessa et al., 2000). Because *pif1 rrm3 S. cerevisiae* cells are viable, the essentiality (Fig. 3A) of the *S. pombe pfh1*$^+$ gene was unexpected.

We purified a truncated form of Pfh1p (Fig. 1) and demonstrated that it had both ATPase (Fig. 2A) and 5' to 3' DNA helicase activity (Fig. 2D). Because alleles with point mutations in the ATP binding motif of Pfh1p were indistinguishable from null alleles (Table II), the ATPase/helicase function of Pfh1p was essential. Cells lacking Pfh1p underwent essentially a complete or nearly complete round of DNA replication (Fig. 4) before arresting as elongated cells (Fig. 3B). Although elongated cell morphology is not a highly specific phenotype, many *S.*

pombe mutants with post initiation defects in DNA replication have an appearance similar to that of *pfh1-D1*::*ura4*$^+$ cells (Gould et al., 1998; Muris et al., 1996).

Although the *S. cerevisiae* Pif1 and Rrm3 proteins affect mitochondrial and rDNA (see introduction), we saw no difference in the structure of mitochondrial or rDNA in mutant versus wild type cells by the criterion of Southern hybridization (Fig. 5). This level of Southern analysis would detect the types of differences seen in the mitochondrial DNA of most mitochondrial deficient *S. cerevisiae* strains but would not detect more subtle changes in abundance or structure of either ribosomal or mitochondrial DNA (Fig. 5B,D). Cytological observations also showed that Pfh1p depleted cells contained mitochondrial DNA (Fig. 3B), and the lethality of the *pfh1-D1*::*ura4*$^+$ mutation was not bypassed by the *ptp1-1* mutation, which allows growth of *S. pombe* cells lacking mitochondrial DNA (Haffter and Fox, 1992). Taken together, these data argue that maintenance of mitochondrial DNA is unlikely to be the essential function of Pfh1p. However, given that Pfh1p was predicted to localize to both mitochondria and nuclei, it might have a non-essential role in mitochondrial DNA metabolism.

Cells lacking Pfh1p exhibited a modest decrease in telomere length (Fig. 5A). As this decrease was manifest by 16 hours, persisted for at least 8 hours, and was not seen with non-telomeric DNA fragments, it was unlikely to be due to DNA degradation in dying cells. The extent of telomere shortening in *pfh1-D1*::*ura4*$^+$ cells was similar to that seen in several mutants defective in both DNA replication and DNA damage checkpoints (Dahlen et al., 1998) or for mutants in either of the two *S. pombe* ATM-like genes (Naito et al., 1998). Despite the role of Pfh1p in maintaining wild type length telomeres, eliminating telomeric DNA did not eliminate the requirement for Pfh1p. Thus, although Pfh1p may affect telomeres, maintenance of telomeric DNA can not be its sole essential function.

Our data suggest that Pfh1p is essential because it plays a vital role in chromosomal DNA replication. The strongest support for this hypothesis is the essentiality (Table II) of the 5' to 3' DNA helicase activity it encodes (Fig. 2). A terminal phenotype similar to that of mutants with defects in DNA replication (Fig. 3B) is consistent with a role for Pfh1p in chromosome replication. An appealing possibility is that Pfh1p, like the *S. cerevisiae* Rrm3p (Ivessa et al., 2000), is required to separate converged replication forks in specific regions of the genome.

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TABLE I: Proteins homologous to *pfh1*$^+$. The predicted protein sequence of *pfh1*$^+$ was compared to the translated DNA database using the default program and parameters of the NCBI server. The expectation score indicates the probability that similarity occurred by chance. All proteins with an expectation score equal to or smaller than 8 x 10^{-50} are shown. *There are no accession numbers for the Pif1-like proteins from *N. crassa* or the basidiomycete *P. chrysosporium*. However, these sequences can be obtained from a search of, respectively, http://www.genome.wi.mit.edu/annotation/fungi/neurospora/ and http://www.jgi.doe.gov/programs/whiterot.htm.

Table II: The ATPase/helicase activity of Pfh1p is essential.

The *pfh1-D*::*ura4*$^+$ *S. pombe* strains 612, which carried a *LEU2* plasmid with *pfh1*$^+$ under the control of its own promoter, or 613, which carried a *LEU2* plasmid with *pfh1*$^+$ under the control of the *nmt1*$^+$ promoter, were transformed with a *his3*$^+$ plasmid carrying either wild type *pfh1*$^+$ or mutant alleles *pfh1*-K337A, or *pfh1*-K337R. The first column indicates the *S. pombe* strain and the identity of the *LEU2* plasmid it contained. Leu$^+$ His$^+$ colonies were streaked two times to medium containing leucine and lacking histidine. The resulting His$^+$ colonies (row 2) were replica plated to determine the fraction of His$^+$ Leu$^-$ cells (row 3). The percentage of colonies that lost the *LEU2* plasmid is also indicated (row 4).

FIGURE LEGENDS

FIGURE 1: Purification of recombinant Pfh1p-h. Amino acids 255 to 789 of the 805 amino acid Pfh1p were fused at the amino terminus to GST and expressed in *S. cerevisiae* from a galactose inducible promoter. Proteins were resolved by 8% SDS-PAGE and detected by Commassie blue staining (A) or, in a separate gel, by immunoblotting with affinity purified anti-

Pfh1p antibodies (B). Lane M, contained prestained protein size markers (NEB). The covalent coupling of the dye to the proteins affects their behavior in SDS-PAGE gels such that the 83 kDa marker has a slower mobility than expected from its mass. The lanes contain total cell extract from an uninduced culture (lane 2), total cell extract from a galactose induced culture (lane 3), 50% ammonium sulfate precipitate from the induced culture (lane 4), extract from induced cells after fractionation on Glutathione sepharose (GST column, lane 5), and extract from induced cells after thrombin digestion to remove the GST moiety and fractionation on Heparin sepharose (lane 6).

FIGURE 2: Recombinant Pfh1p-h has single strand DNA stimulated ATPase and 5' to 3' DNA helicase activities. Truncated Pfh1p (called Pfh1p-h; h for helicase) was purified as shown in Fig. 1 and used in enzyme assays. (A) ATPase activity assay. Reactions were carried out in ATPase buffer as described in Materials and Methods, the products were developed in polyethylimine (PEI) cellulose plate and visualized on a Molecular Dynamics Phosphoimager. Lane 5 contains γ-P^{32} ATP, M13 single strand DNA, $Mg2^{+}$, and recombinant Pfh1p-h. The other lanes were the same except lane 1 contained recombinant Pif1p, prepared as described in (Zhou et al., 2000), in place of Pfh1p-h; lane 2, contained BSA in place of Pfh1p-h; lane 3 had no $Mg2^{+}$, and lane 4 had no single strand DNA. (B) Helicase activity assay and (C) polarity assay. Kinase labeled 25-mer oligonucleotide, kinase labeled 36-mer oligonucleotide and single-stranded M13 DNA were used to make the substrate for the helicase assays. The products were resolved in 10% PAGE and visualized on a Molecular Dynamics Phosphoimager. Lane 1, shows heat denatured substrate. Lane 5 contains γ-P^{32} ATP, Mg^{2+}, and recombinant Pfh1p-h. The other lanes are the same except lane 2 contains BSA instead of Pfh1p-h; lane 3 lacks ATP; lane 4 lacks Mg^{2+}, and lane 6 contains recombinant Pif1p in place of Pfh1p-h.

FIGURE 3: Phenotypes of cells lacking Pfh1p. A. The *pfh1*$^+$/*pfh1-D1*::*ura4*$^+$ diploid strain was sporulated, and the four haploid spore progeny from each of seven tetrads, were separated on rich medium, arranged top to bottom, and allowed to grow for three days at 30° C. Viability segregated 2^+:2^- and viable spores were always Ura$^-$. Spores that failed to form visible colonies often divided one or, more rarely, several times to form microcolonies containing up to ten cells. B: A *pfh1*$^+$/*pfh1-D1*::*ura4*$^+$ diploid strain was sporulated. Spores were germinated and grown in medium lacking uracil, in which *pfh1*$^+$ cells can not grow owing to their being Ura$^-$. DNA was stained using the fluorescent dye Yo-Yo 2 (Molecular Probes, Eugene, OR), and cells were examined by fluorescence microscopy at various times after sporulation. The sample shown was taken 28 hours after sporulation. At 28 hrs, the final cell number was 2-3 times higher than that of the starting culture, indicating that many *pfh1-D1*::*ura4*$^+$ cells had divided one or two times. The speckled fluorescent dots are mitochondria (Haffter and Fox, 1992). C. Analysis is as in B, except that a wild type diploid was sporulated, and cells were germinated and grown in medium containing uracil.

FIGURE 4. Cells lacking Pfh1p arrest with a 2C DNA content. Spores derived from a *pfh1-D1*::*ura4* /*pfh1*$^+$ heterozygote were inoculated into germination medium lacking uracil so that only *pfh1-D1*::*ura4*$^+$ mutant spores could germinate. Control spores derived from a *pfh1*$^+$ /*pfh1*$^+$ homozygote (wild type) were inoculated into germination medium containing uracil such that all spores could germinate. Samples of cells were harvested at various time points after inoculation, stained with propidium iodide and analyzed by FACS for DNA content.

FIGURE 5. Deletion of *pfh1*$^+$ is associated with shortening of *S. pombe* telomeres. DNA was isolated from *pfh1*$^+$ and *pfh1-D1*::*ura4*$^+$ cells after 16, 20 or 24 hours of germination. Panel A contains DNA digested with *Apa*I (left lanes) or *Hin*fI (right lanes), and probed with a *Taq*I fragment from pSPT16 that contains *S. pombe* telomeric sequences (but not sub-telomeric sequences). For *Apa*I, the wild type and mutant samples are grouped whereas mutant and wild type samples are alternated for the *Hin*fI digested samples. In *Apa*I digested DNA, there is an *Apa*I site very near the tract of telomeric repeats in 5 of the 6 telomeres, such that the broad band of ~300 bps contains most of the telomeres. *Hin*fI digested DNA places five of the six telomeres on fragments of differing sizes. Panels B, C, and D are the same *Hin*fI digested samples from panel A, after analysis with the following probes: (B) a 2.4 kb PCR fragment from *S. pombe* rDNA. This probe hybridizes to multiple *Hin*fI fragments, seven of which are smaller than 360 bp. Shown are the three largest hybridizing fragments of 1077 bp and a doublet of 825 and 856 bp. (C) a 288 bp PCR fragment from the *S. pombe rhp6*$^+$ gene, which hybridizes to a unique, non-telomeric 2.1 kb *Hin*fI fragment. (D) *S. pombe* mitochondrial DNA from plasmid pDG3 (Del Giudice, 1981) which hybridizes to multiple *Hin*fI fragments. The positions of molecular weight markers are indicated.

Supplemental Material: Sequence of the *S. pombe pfh1*$^+$ gene. The transcription initiation site is in bold, underscored and indicated by an arrow. Upstream out of frame AUG's are indicated by an M and are underlined. The first AUG of the *pfh1*$^+$ ORF is underlined and in bold. Intron junctions are noted by In, and splicing signals are underlined and in bold. Nucleotides in bold that are underlined by dotted lines are matches to MCB (*Mlu* cell cycle box) sequence. The amino acid residues in each of the seven helicase motifs are underscored by dotted lines and denoted by the appropriate roman numeral (the seven motifs are named I, Ia, II –

VI). The lysine residue in the ATP binding pocket of motif I that was mutated is boxed. Motif II is split by an intron. The brackets indicate the region of Pfh1p that was expressed as a GST fusion and used for the biochemical assays in Fig. 1 and 3. The region deleted in the *pfh1-D1*::*ura4*$^{+}$ deletion/insertion mutation is denoted by Δ's. The sequence ends with the *Cla*I site (denoted with italics) used to clone *pfh1*$^{+}$ into plasmid pVS110 (see methods).

Table 1: Proteins homologous to *pfh1*$^{+}$.

Accession Number*	Gene	Expectation Score
Contig 1.441	*N. crassa*	1×10^{-149}
X05342	*S. cerevisiae PIF1*	1×10^{-117}
U00062	*S. cerevisiae RRM3*	1×10^{-105}
Scaffold_152	*P. chrysosporium*	1×10^{-101}
AB015041	*C. elegans*	5×10^{-76}
AE003579	*D. melanogaster*	2×10^{-70}
AF108138	*H. sapiens*	1×10^{-61}
AF083915	*Chilo iridescent virus*	9×10^{-56}
X74788	*C. maltosa*	4×10^{-54}
Scaffold_162	*P. chrysosporium*	2×10^{-54}
Contig 1.464	*N. crassa*	8×10^{-50}

Table 2: The ATPase/helicase activity of Pfh1p is essential

S. pombe strain/*LEU2* plasmid	# His^+ colonies	# His^+Leu^- colonies	% colonies that lost *LEU2* plasmid
612/*pfh1*$^+$	188	140	74.4
612/*pfh1*-K337R	320	0	<0.3
612/*pfh1*-K337A	309	0	<0.3
613/*pfh1*$^+$	204	200	98.0
613/*pfh1*-K337R	388	0	<0.2
613/*pfh1*-K337A	147	0	<0.7

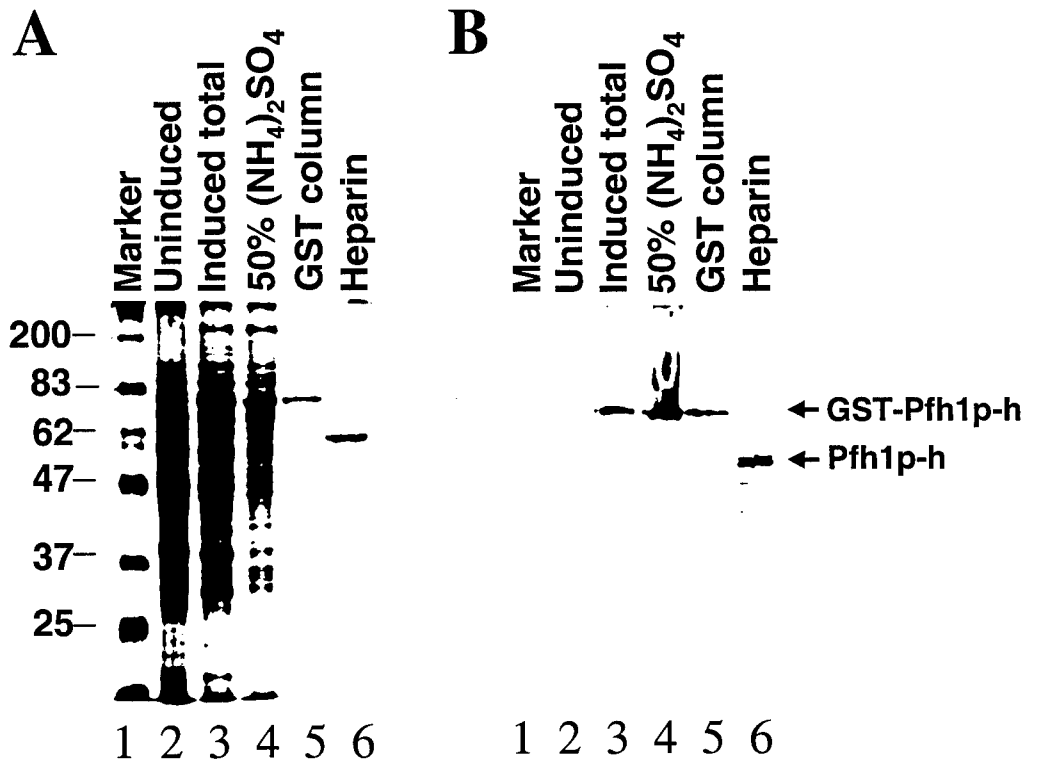


Fig. 1 (Zhou et al.)

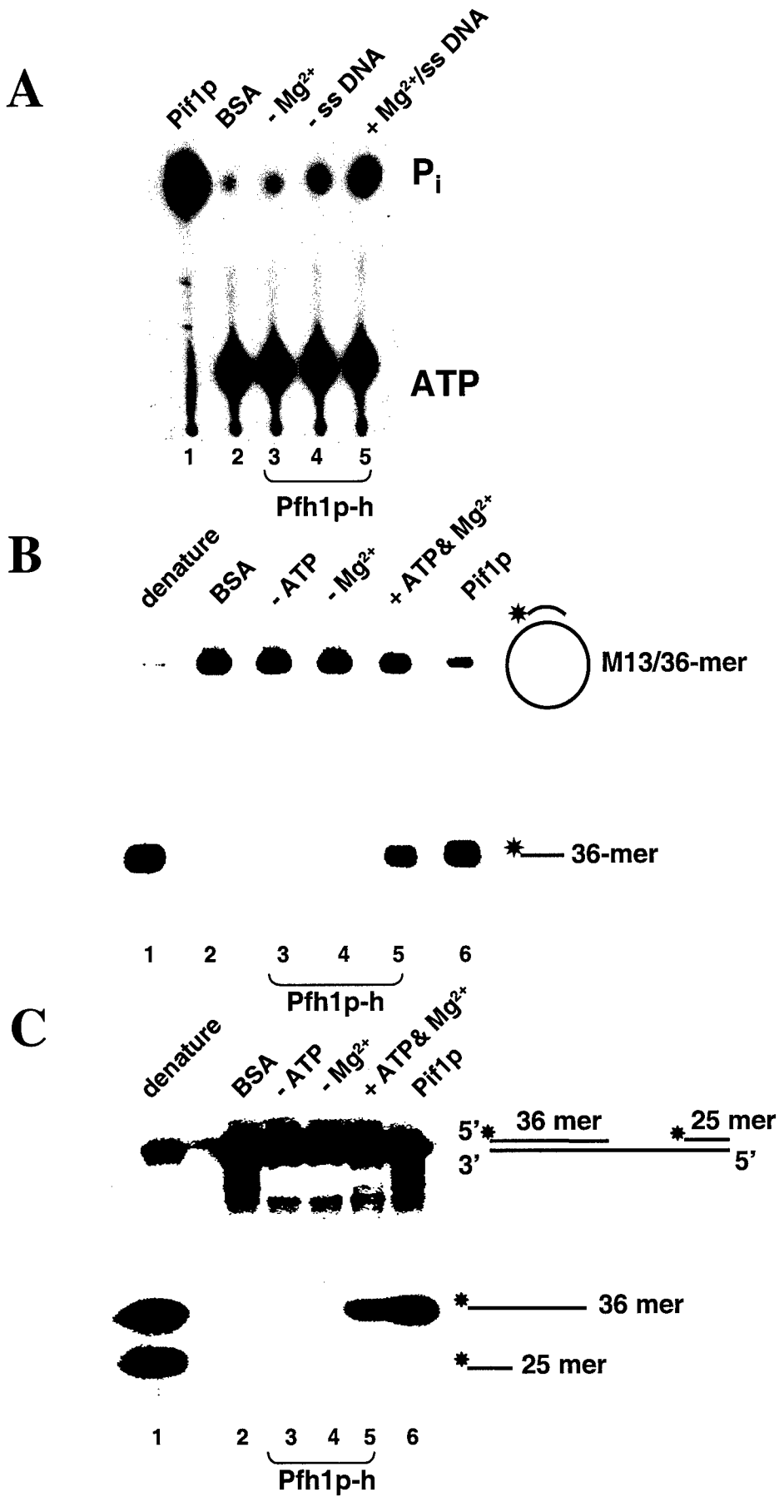
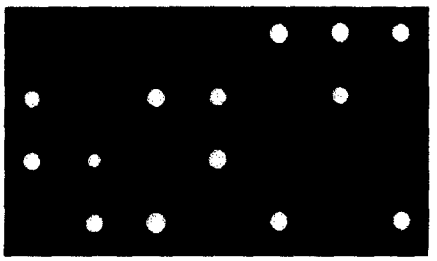


Fig. 2 (Zhou et al.)

A.



B. *rph1⁻-D1::ura4⁺*

C. *rph1⁺*

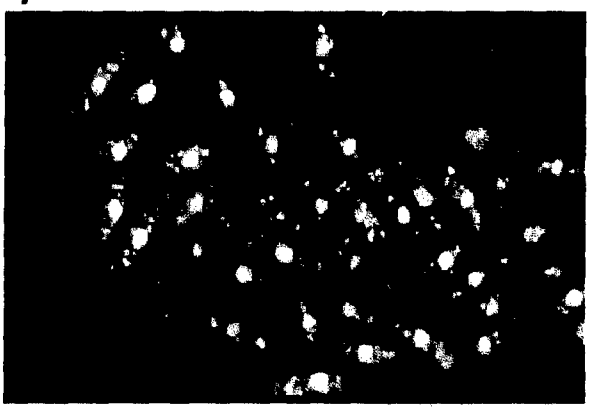


Fig. 3 (Zhou et al.)

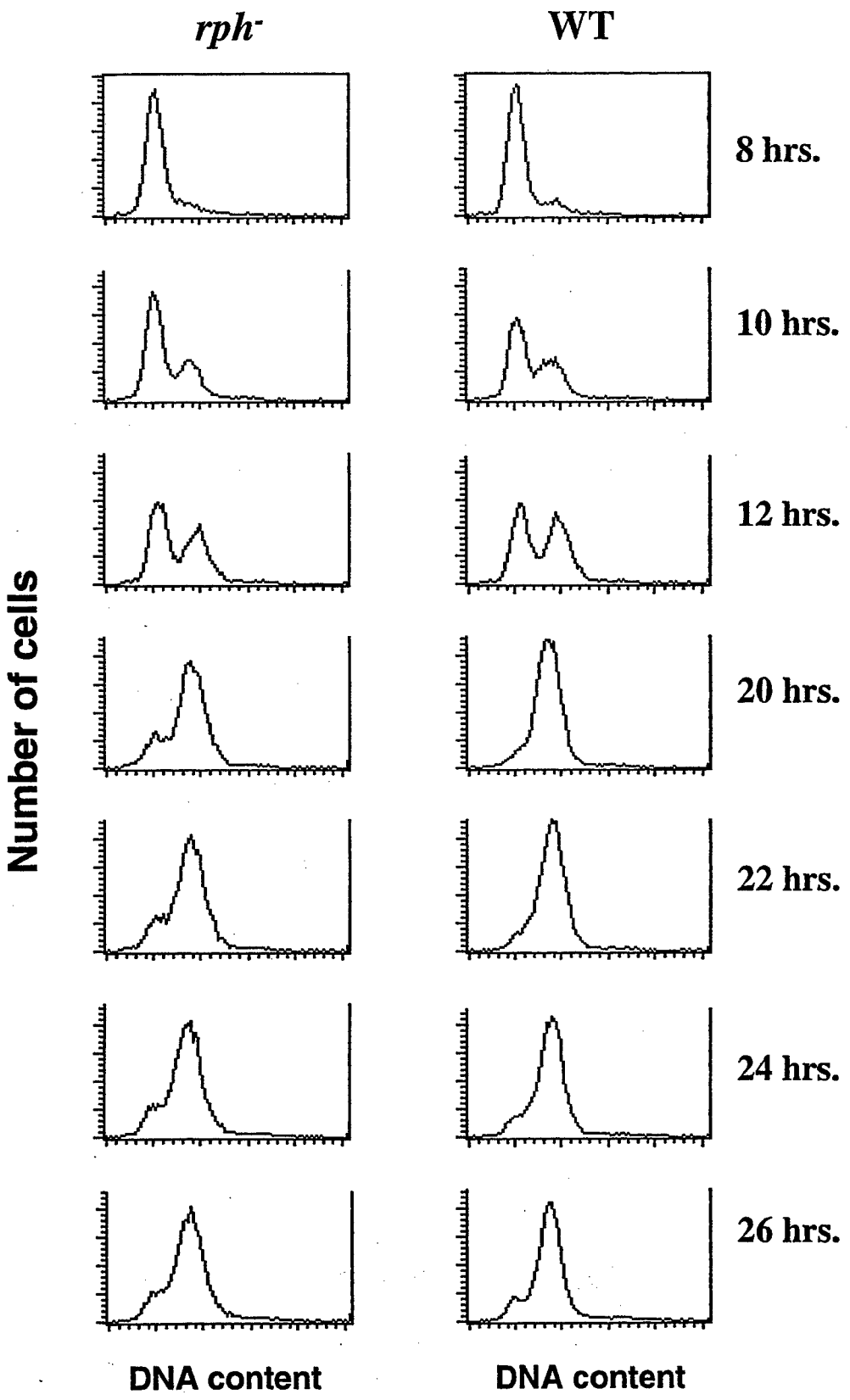


Figure 4 (Zhou et al.,)

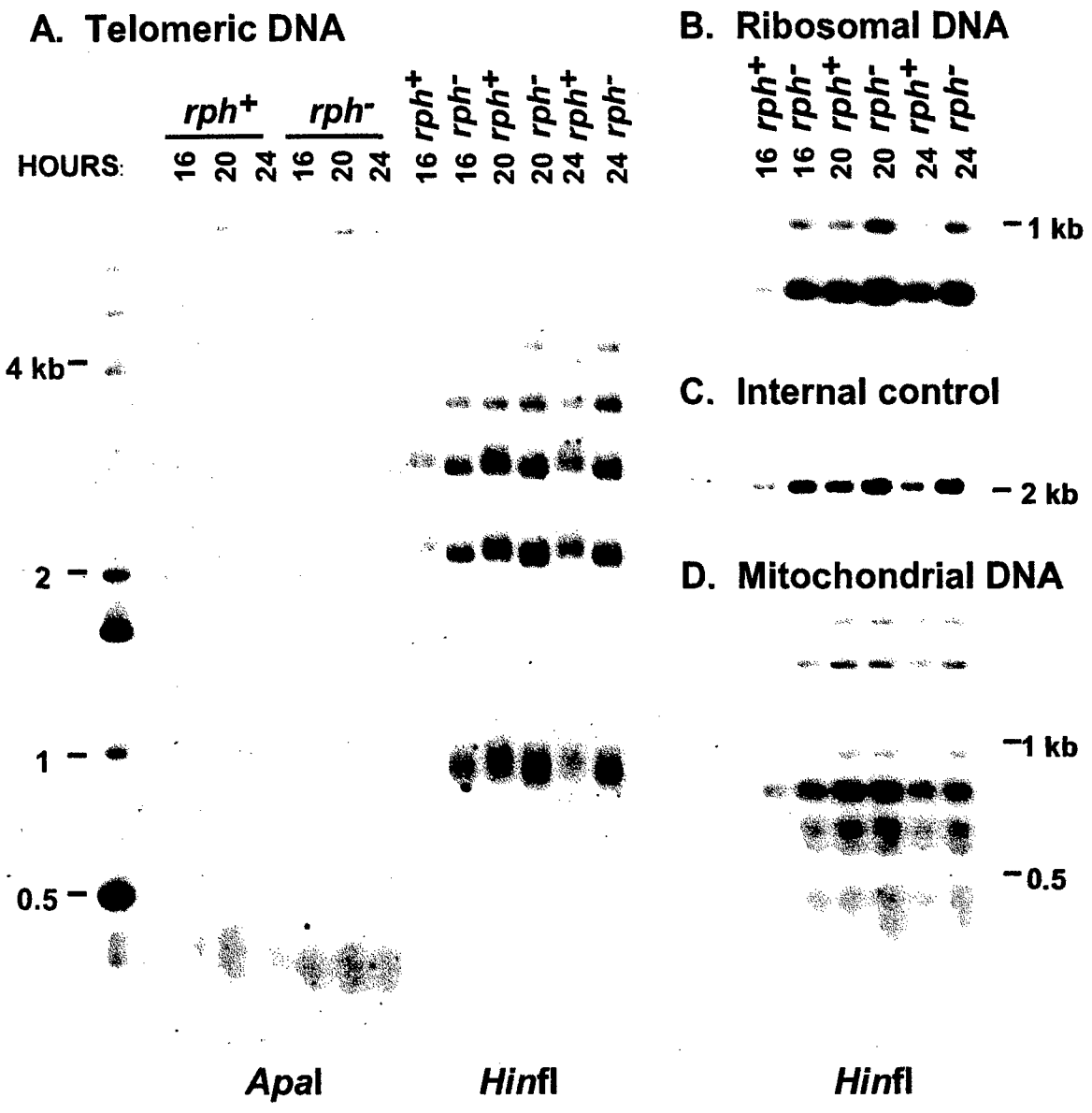


Fig. 5 (Zhou et al.)

The *Saccharomyces* telomere-binding protein Cdc13p interacts with both the catalytic subunit of DNA polymerase α and the telomerase-associated Est1 protein

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Saccharomyces **telomeres consist of ~350 bp of $C_{1-3}A/TG_{1-3}$ DNA. Most of this ~350 bp is replicated by standard, semiconservative DNA replication. After conventional replication, the $C_{1-3}A$ strand is degraded to generate a long single strand TG_{1-3} tail that can serve as a substrate for telomerase. Cdc13p is a single strand TG_{1-3} DNA-binding protein that localizes to telomeres in vivo. Genetic data suggest that the Cdc13p has multiple roles in telomere replication. We used two hybrid analysis to demonstrate that Cdc13p interacted with both the catalytic subunit of DNA polymerase α, Pol1p, and the telomerase RNA-associated protein, Est1p. The association of these proteins was confirmed by biochemical analysis using full-length or nearly full-length proteins. Point mutations in either *CDC13* or *POL1* that reduced the Cdc13p–Pol1p interaction resulted in telomerase mediated telomere lengthening. Over-expression of the carboxyl terminus of Est1p partially suppressed the temperature sensitive lethality of a *cdc13-1* strain. We propose that Cdc13p's interaction with Est1p promotes TG_{1-3} strand lengthening by telomerase and its interaction with Pol1p promotes $C_{1-3}A$ strand resynthesis by DNA polymerase α.**

[*Key Words*: Telomere replication; telomerase; DNA polymerase; *CDC13*; *POL1*; *EST1*]

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In most eukaryotes, telomeres, the physical ends of chromosomes, consist of simple repeated DNA sequences and their associated proteins. Chromosomes in *Saccharomyces cerevisiae* end with ~350 bp of duplex $C_{1-3}A/TG_{1-3}$ DNA. In organisms where the structure at the very end of the chromosome has been examined, the G-rich strand is extended to form a single-strand tail. Telomere-associated proteins include proteins that directly bind to either duplex or single-stranded telomeric DNA as well as those that are brought to the telomere by protein–protein interactions. The major *Saccharomyces* telomere protein is the duplex $C_{1-3}A/TG_{1-3}$ binding protein Rap1p (Conrad et al. 1990; Wright et al. 1992; Wright and Zakian 1995). The Cdc13p protein, which binds ss TG_{1-3} DNA in vitro (Lin and Zakian 1996; Nugent et al. 1996), is also localized to telomeres in vivo (Bourns et al. 1998). Unlike Rap1p, Cdc13p does not bind to internal tracts of $C_{1-3}A/TG_{1-3}$ DNA (Bourns et al. 1998), suggesting that its association with the telomere is due to its ability to bind ss TG_{1-3} DNA.

Telomeres are required for the complete replication of linear chromosomes. After replication by a conventional DNA polymerase, removal of the terminal RNA primer leaves an 8–12 base gap at the 5′ end of newly replicated DNA molecules, a gap that can not be repaired by a conventional DNA polymerase. In most eukaryotes, including yeast, the end replication problem is solved by telomerase, a reverse transcriptase that is able to extend the short G-tail left after RNA primer removal, using an integral RNA as its template. There are at least five *Saccharomyces* genes that are required for the telomerase pathway in vivo, *EST1*, *EST2*, *EST3*, *TLC1*, and *CDC13* (for review, see Nugent and Lundblad 1998). Mutation of any one of these genes causes a gradual loss of telomeric DNA or *est* (ever shorter telomere) phenotype, consistent with incomplete replication of chromosome ends. However, the products of only two of these genes, *TLC1*, which encodes the RNA component of telomerase, and *EST2*, the gene for its catalytic component, are essential for telomerase activity in vitro (for review, see Nugent and Lundblad 1998). Est1p is associated with *TLC1* RNA (Lin and Zakian 1995; Steiner et al. 1996) and also binds ss TG_{1-3} DNA in vitro (Virta-Pearlman et al. 1996; Zhou et al. 2000). *EST1* mutations that reduce Est1p binding to telomerase RNA have an *est* phenotype (Zhou et al. 2000). Given that Cdc13p, an in vivo telomere-binding protein (Bourns et al. 1998), is not required for telomerase activity in vitro (Lingner et al. 1997a), an appealing model is that Cdc13p recruits telomerase to the telo-

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mere. This model is supported by the demonstration that a Cdc13p–Est2p fusion protein bypasses the requirement for both Est1p and Est2p (Evans and Lundblad 1999).

Analysis of telomere replication intermediates revealed an unexpected step in telomere replication. Conventional semiconservative replication of the telomeric $C_{1\text{-}3}A/TG_{1\text{-}3}$ repeats occurs late in S phase (Wellinger et al. 1993a). After conventional replication is complete, both ends of individual chromosomes acquire 50–100 base ss $TG_{1\text{-}3}$ tails (Wellinger et al. 1993a, b). Because these G-tails are detected in cells lacking telomerase (Dionne and Wellinger 1996; Wellinger et al. 1996), they must be generated by C-strand degradation. As Cdc13p binds efficiently to both 43 and 270 base $TG_{1\text{-}3}$ tails in vitro (Lin and Zakian 1996), these long G-tails are suitable substrates for Cdc13p; and indeed, genetic data reveal that Cdc13p regulates C-strand degradation. When *cdc13-1* cells are grown at restrictive temperatures, they accumulate ssDNA that extends from the telomere many kilobases towards the center of the chromosome with the C-strand being preferentially degraded (Garvik et al. 1995). C-strand degradation was unanticipated as it exacerbates the end problem of replication by creating a large gap at both ends of individual chromosomes.

It is unclear when or how often telomerase acts on a given chromosome end. As the rate of telomerase lengthening is inversely proportional to telomere length (Marcand et al. 1999), it is possible that only short telomeres are substrates for telomerase. Although telomerase activity can be detected in extracts prepared from cells throughout the cell cycle, formation of new telomeres is restricted to late G2/M phase (Diede and Gottschling 1999). Regardless of whether or not a given telomere is acted upon by telomerase, its C-strand must be resynthesized as long ss $TG_{1\text{-}3}$ tails are not detected in post S/G2 phase cells (Wellinger et al. 1993a,b). Presumably, C-strand resynthesis is accomplished by a conventional DNA polymerase, although the polymerase that carries out this step has not been identified, nor is it known how DNA polymerase is recruited to the transient G-tail. After the 8–12 base RNA that is expected to prime C-strand resynthesis is removed, newly replicated telomeres should bear an 8–12 base ss $TG_{1\text{-}3}$ tail. As duplex telomeric DNA bearing a five or 10 base $TG_{1\text{-}3}$ tail is bound by Cdc13p in vitro (Lin and Zakian 1996), newly replicated telomeres are suitable substrates for Cdc13p.

Cdc13p appears to be a key regulatory protein in telomere replication as it functions at two critical steps in yeast telomere metabolism, C-strand degradation and G-strand elongation. To gain insight into how Cdc13p regulates these steps, we identified proteins that interact with Cdc13p in vivo. We found that Cdc13p interacts with the catalytic subunit of DNA polymerase α, Pol1p, and Est1p, by both two hybrid and biochemical criteria. Mutations in either *CDC13* or *POL1* that disrupt the Cdc13p–Pol1p interaction affect telomere length. Overexpression of the portion of Est1p that interacted with Cdc13p by two hybrid criteria partially suppressed the temperature sensitive lethality of a *cdc13-1* strain. The interaction of Cdc13p with Est1p provides additional support for a model in which Cdc13p promotes the telomerase pathway by recruiting Est1p to the telomere. We propose that Cdc13p's interaction with Pol1p promotes C-strand resynthesis.

Results

Cdc13p interacts with Pol1p by two hybrid criteria

A two hybrid screen (Fields and Song 1989; Gyuris et al. 1993) was used to identify proteins that interact with Cdc13p in vivo. The first 600 amino acids of the 924 amino acid Cdc13p were expressed as a fusion to the LexA DNA binding domain and used as bait in the yeast strain YEM1α, which carries two reporter genes, *LEU2* and *LacZ* (Fig. 1; the fusion polypeptide encoded by this plasmid is called Cdc13Np). The amino acid changes that confer the *cdc13-1* and *cdc13-2* phenotypes are both contained within this amino-terminal segment of Cdc13p (Lin and Zakian 1996; Nugent et al. 1996), and this region is sufficient to target Cdc13p to telomeres in vivo (Bourns et al. 1998). A library that produces galactose-inducible proteins as fusions of polypeptides encoded by segments of yeast chromosomal DNA and a transcriptional activation domain from *Escherichia coli* (Watt et al. 1995) was introduced into YEM1α cells carrying the bait plasmid. If a library plasmid produces a fusion protein that interacts with Cdc13Np, it will activate the *LEU2* gene, allowing growth on media lacking leucine. A fusion protein that interacts strongly with Cdc13Np will also activate the *lacZ* gene, generating blue color when cells are assayed for β-galactosidase ac-

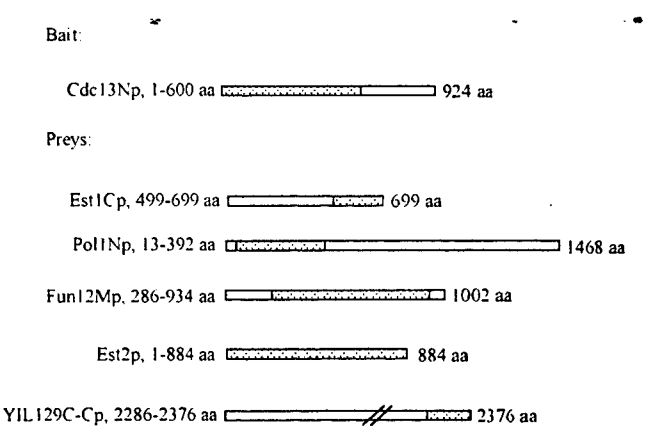


Figure 1. Polypeptides used in the two-hybrid assay. Dotted regions indicated the portion of Cdc13p expressed from the bait plasmid pEG202 and the portions of proteins expressed from the prey vector pJG4-5 (not to scale). The fusion of the amino-terminal region of Cdc13p with the LexA DNA binding domain is referred to as Cdc13Np in the text. Prey proteins are similarly named; e.g., Pol1Np refers to the fusion of 379 amino acids from near the amino-terminus of Pol1p to the activation domain peptide. The amino acid numbers after the name of each protein indicates the specific amino acids expressed from the two-hybrid vector. The numbers to the right of the schematic for each protein indicate the number of amino acids in the full-length protein.

tivity. Colonies expressing interacting fusion proteins were selected by their ability to grow on galactose media lacking leucine (Fig. 2A) and then screened for production of blue color by a filter LacZ assay (Breeden and Nasmyth 1985).

From a screening of more than 5×10^6 colonies, we obtained three positive clones. DNA sequence analysis revealed that the plasmids contained amino acids 13–392 from the 1468 amino acid Pol1p, amino acids 286–934 from the 1002 amino acid Fun12p, and amino acids 2286–2376 from the 2376 amino acid YIL129C (Fig. 1; the fusion polypeptides encoded by these prey plasmids were named Pol1Np, Fun12Mp, and YIL129C-Cp). The essential gene *FUN12* encodes a protein that is 27% identical to the *E. coli* IF2 protein and, like its bacterial counterpart, is involved in the initiation of protein synthesis (Choi et al. 1998). Virtually nothing is known about the function of YIL129C. Pol1p is the catalytic subunit of DNA polymerase α, one of three yeast polymerases required for chromosomal DNA replication (Waga and Stillman 1998). Given its central role in DNA replication and the fact that certain *POL1* mutations cause telomere lengthening (Carson and Hartwell 1985; Adams and Holm 1996), we decided to focus on the interaction between Pol1p and Cdc13p.

By the criteria of a two hybrid assay, the interaction of Pol1Np with Cdc13Np was both specific and strong (Fig. 2A). Activation was seen only on galactose medium, not for glucose-grown cells, and required the presence of both the bait and prey plasmids (negative controls 1 and 2; Fig. 2A). The *POL1* polypeptide did not activate in cells expressing other baits, such as LexA::cRafp (a gift from E. Golemis) (Fig. 2A, cRaf). Cells expressing the interacting Pol1Np and Cdc13Np polypeptides grew as well on plates lacking leucine as the strongly interacting proteins Rpb4p and Rpb7p, two subunits of RNA polymerase II (Khazak et al. 1995) (Fig. 2A, positive control), and produced a similar amount of β-galactosidase by a LacZ filter assay (data not shown).

Full-length Cdc13p and Pol1p interact by biochemical criteria

Immunoprecipitation (IP) was used to determine if the interaction between Cdc13Np and Pol1Np detected by two-hybrid analysis reflected an in vivo association of full-length proteins (Fig. 2B). Extracts were prepared from cells carrying the endogenous copies of both genes expressed from their own promoters with the only modification being the introduction of three MYC epitopes at the Cdc13p carboxyl terminus. Cells with the MYC_3-tagged *CDC13* allele had a normal growth rate and no detectable change in telomeric DNA (data not shown). Monoclonal anti-MYC antibody was used to immunoprecipitate MYC_3-tagged Cdc13p. The precipitate was analyzed by Western blotting using both a monoclonal anti-Pol1p (a generous gift from Dr. P. Plevani) and anti-MYC antibodies. MYC_3–Cdc13p was readily detected in the anti-MYC immunoprecipitate (Fig. 2B). Cdc13p appeared as a doublet, because of its existing in

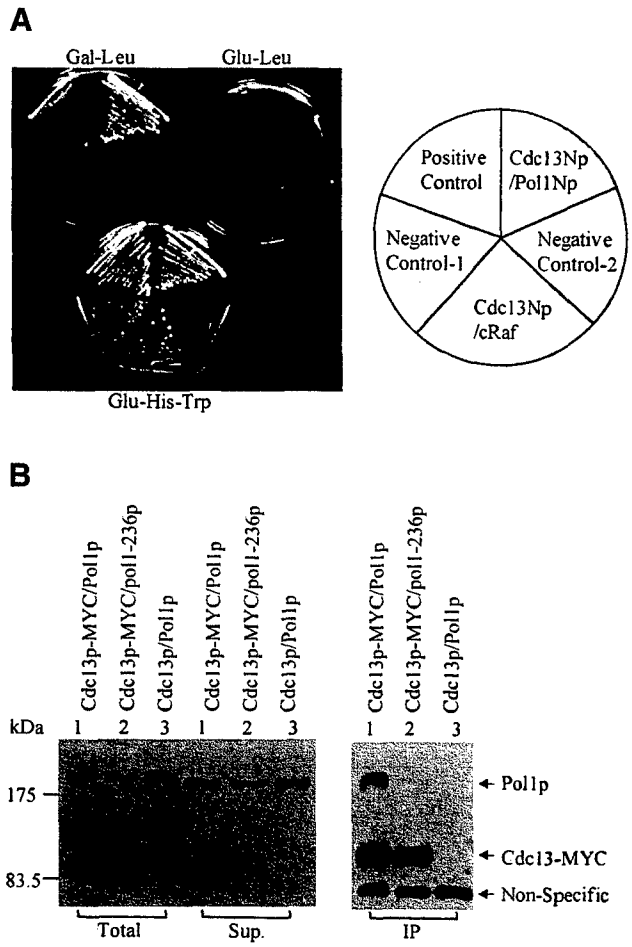


Figure 2. Cdc13p interacted with Pol1p, the catalytic subunit of DNA polymerase α. (*A*) Cells expressing prey and/or bait proteins were streaked on galactose plates lacking leucine (Gal–Leu). Galactose induces expression of prey proteins and growth in the absence of leucine requires interaction of bait and prey polypeptides. Alternatively, cells were streaked on glucose plates lacking leucine (Glu-Leu) where prey proteins are not expressed or glucose plates lacking histidine and tryptophan (Glu-His-Trp), which selects for maintenance of both the prey and bait plasmids but not for interaction of their products. The positive control was cells expressing two subunits of RNA polymerase II, Rpb4p and Rpb7p. The negative controls were cells expressing Cdc13Np and carrying the empty prey vector pJG4-5 (Negative Control–1), cells expressing Pol1Np and carrying the empty bait vector pEG202 (Negative Control-2), and cells expressing Cdc13Np and a LexA::cRafp fusion protein (gift from E. Golemis) (Cdc13Np/cRaf). (*B*) Extracts from cells carrying an MYC_3-tagged Cdc13p were immunoprecipitated by monoclonal anti-MYC antibody immobilized on protein-A and protein-G beads. The extract prior to immunoprecipitation (Total), the supernatant from the immunoprecipitate (Sup.), and the immunoprecipitate (IP) were analyzed by Western blotting using both anti-MYC and antiPol1p monoclonal antibodies. Although Cdc13p was not visible in the total cell extract in this gel, it was detectable when more protein was loaded. Extracts were prepared from cells containing 3XMYC (lanes *1,2*) or untagged (lane *3*) Cdc13p. Cells had either the wild-type *POL1* (lanes *1,3*) or the *pol1-236* allele (lane *2*). The nonspecific band was detected by the anti-MYC serum.

different phosphorylated states in vivo (A. Taggart and V.A. Zakian, unpubl.) (Fig. 2B, panel IP). Pol1p was detectable in the anti-MYC immunoprecipitate (Fig. 2B, lane 1 IP) but only if the extract was prepared from a strain with an MYC_3-tagged Cdc13p (Fig. 2B, cf. lanes 1 and 3, IP). We conclude that full-length Pol1p and Cdc13p interact in vivo.

Identification of Pol1Np mutations that reduce its ability to interact with Cdc13Np

To identify point mutations in Pol1Np that disrupted the Cdc13p–Pol1p interaction, we used PCR to mutagenize the 379 amino acid fragment of Pol1p identified in the two-hybrid screen (Leung et al. 1989; Zhou et al. 1991). The mutagenized fragments were then assessed for their ability to interact with Cdc13Np in the two-hybrid assay. Transformants that produced white colonies in the lacZ filter assay were chosen for further analysis. Out of ~10^6 colonies, three mutants gave white or pale blue colored colonies in the LacZ filter assay and produced full-length polypeptides by Western analysis (Fig. 3B). As each of these mutants produced normal amounts of Pol1Np, their failure to interact in the two hybrid assay was not attributable to failure in protein expression. DNA sequencing showed that each of the three had a single amino acid substitution: aspartic acid to asparagine at residue 236 (D236N), glutamic acid to lysine at residue 238 (E238K), and proline to threonine at residue 241 (P241T), respectively (Fig. 3A). Mutations were named *pol1-236, 238,* and *241* according to the amino acid residue that was mutated.

To estimate the extent of loss of the Pol1Np–Cdc13Np two-hybrid interaction in each of the *pol1* mutants, a quantitative liquid lacZ assay was performed (Guarente 1983). Using this assay, interaction with Cdc13Np was essentially abolished in *pol1-236* (D236N), reduced ~50% in *pol1-238* (E238K) and reduced ~90% in *pol1-241* (P241T) (Fig. 3C).

Mutations in Pol1p that disrupt the interaction with Cdc13p result in longer telomeres

To determine the importance of the Cdc13p–Pol1p interaction, each of the three *POL1* mutations that reduced or eliminated this interaction by two-hybrid criteria was introduced into the chromosomal copy of *POL1*, using the integration plasmid pCM1 (Lucchini et al. 1988). In these *pol1* haploid isolates, the mutant allele was the only copy of *POL1*, and Pol1p was expressed from its own promoter. Strains carrying the *pol1-236, 238,* or *241* alleles grew as well as wild-type cells at 23°C, 30°C and 37°C (data not shown). Thus, none of the mutant alleles eliminated an essential function of Pol1p.

To determine if telomere length is altered in the *pol1* mutant strains, DNA was prepared from wild-type and mutant cells, digested with *Xho*I, and analyzed by Southern blotting using a telomere probe (Fig. 4A). *Xho*I digestion generates a ~1.3-kb terminal fragment from Y′-bearing telomeres, which make up about two-thirds of the telomeres in yeast, and multiple larger sized bands from X-bearing telomeres. Each of the *pol1* mutants caused an increase in the length of X′ and Y′ telomeres (Fig. 4A). Cells with the *pol1-236* allele, the allele that essentially eliminated the Pol1Np–Cdc13Np interaction by two-hybrid criteria (Fig. 3C), had telomeres that averaged 150-bp longer than wild type (Fig. 4A, lanes 2,3). The *pol1-238* cells had telomeres that were ~40 bp longer than wild type (Fig. 4A, lanes 5,6) and an ~50% reduction in the Pol1Np–Cdc13Np interaction (Fig. 3C). The *pol1-241* cells had telomeres that were ~90 bp longer than wild type (Fig. 4A, lanes 8,9) and an ~90% reduction in the

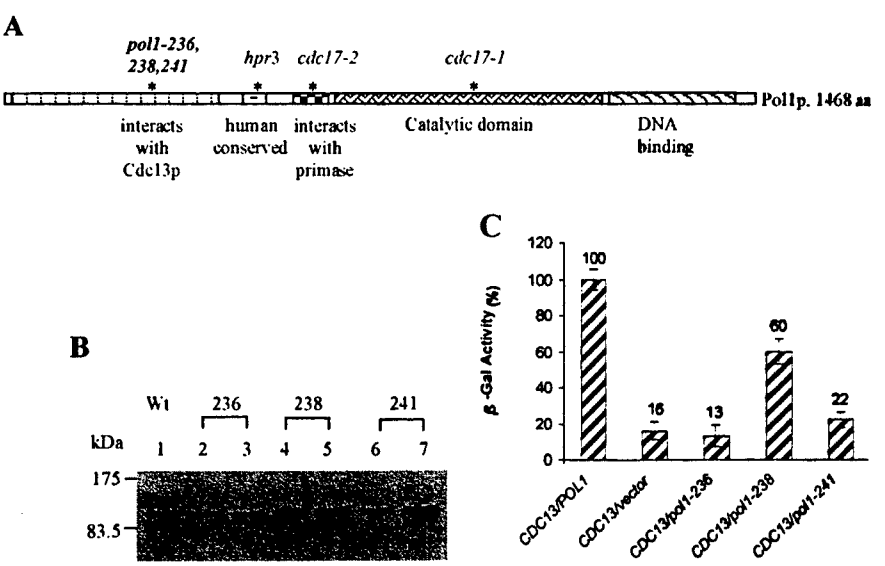


Figure 3. Analysis of *pol1* alleles that disrupt interaction of Pol1p with Cdc13p. (*A*) Positions of functional domains and locations of various mutations within Pol1p are indicated. Mutations in bold disrupted Pol1p interaction with Cdc13p. Positions or regions containing mutations characterized in other labs are also shown: *hpr3* (G439E), *cdc17-2* (G637D), *cdc17-1* (G904D) (Pizzagalli et al. 1988; Lucchini et al. 1990). (*B*) The wild-type (lane *1*) or mutant (lanes *2–7*) proteins were expressed from the pJG4-5 vector under control of a *GAL1* promoter. The allele number of the expressed protein is indicated above the lanes. Proteins expressed from pJG4-5 are fused to a transcriptional activation domain and an HA epitope. Cell extracts were analyzed from two different isolates for each mutation using Western blotting with an anti-HA antibody. (*C*) Extracts were produced from strains expressing both Cdc13Np and either the pJG4-5 vector alone or pJG4-5 with the wild-type *POL1* segment or one of the point mutants, as indicated below. Data are the average of three independent β-galactosidase measurements. Error bars, S.D.

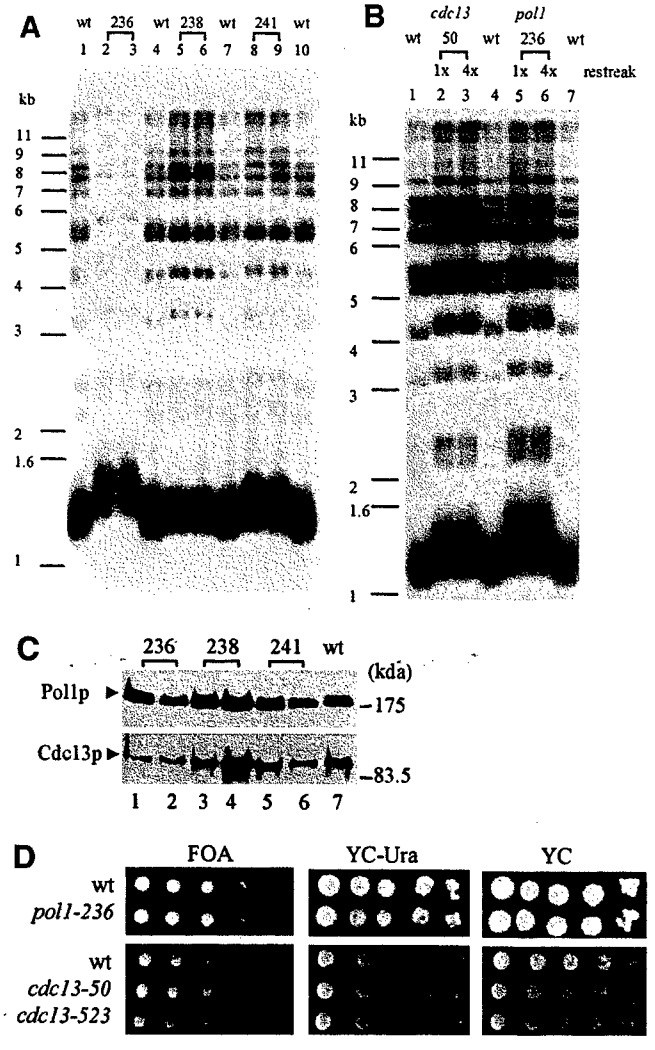


Figure 4. The *pol1* alleles that disrupt Pol1p-Cdc13p interaction cause telomere lengthening. (*A*) Genomic DNA was isolated from wild type and two independent isolates for each of the three *pol1* alleles (allele numbers are above the lanes), digested with *Xho*I and analyzed by Southern hybridization using a telomeric probe. (Lanes *2, 3*) *pol1-236* cells; (lanes *5, 6*) *pol1-238* cells; (lanes *8, 9*) *pol1-241* cells. (Lanes *1, 4, 7, 10*) DNA from an otherwise isogenic wild-type strain. (*B*) Cells of the indicated genotype were streaked on complete media right after meiosis and allowed to form colonies. DNA was prepared from colonies after the first restreak (~55 divisions postgermination) and after four restreaks (~130 generations). DNA was analyzed as described for panel *A*. DNA is from wild type (lanes *1, 4, 7*), *cdc13-50* cells (lane *2*, 55 divisions; lane *3*, 155 divisions), and *pol1-236* cells (lane *5*, 55 divisions; lane *6*, 155 divisions). (*C*) Western analysis was carried out using DNA from *POL1* or *pol1-236*, -238, and -241 strains expressing 3XMYC- Cdc13p (mutant allele indicated above the lanes; extracts from two independent mutant isolates are shown for each mutant allele). Extracts were analyzed by SDS–PAGE and Western blotting using the anti-Pol1p serum (*top*) or anti-myc serum (*lower panel*). The minor differences in protein levels were not reproducible. (*D*) To measure telomere position effect, ten-fold serial dilutions of otherwise isogenic strains that were either wild type (wt) or contained the indicated mutations and having *URA3* next to the left telomere of chromosome VII were spotted onto plates containing complete medium plus FOA, complete medium lacking uracil (YC-uracil), or complete medium (YC).

Pol1Np–Cdc13Np interaction (Fig. 3C). The increase in telomere length seen in these mutants was not progressive, as telomeres were equally long at ~55 and ~130 divisions after sporulation (Fig. 4B). This telomere lengthening was telomerase-mediated as lengthening was not seen in a *tlc1 pol1-236* double mutant strain (data not shown). As Western analysis showed that each of the mutant alleles produced essentially wild-type levels of both Pol1p and Cdc13p (Fig. 4C), the differences in telomere lengths are unlikely to be attributable differences in the abundance of either protein.

Telomere position effect, (TPE), refers to the fact that genes placed near a yeast telomere are transcriptionally repressed (Gottschling et al. 1990). Certain *POL1* alleles that affect the catalytic activity of polymerase α cause telomere lengthening, loss of TPE, and altered G-tail metabolism (Adams-Martin et al. 2000). To monitor TPE, the *URA3* gene was placed next to the left telomere of chromosome VII in the *pol1-236* strain and the fraction of cells able to grow on FOA medium determined. As the *pol1-236* strain had the same fraction of FOA^R cells as an isogenic wild-type control (Fig. 4D), TPE was not affected by the *pol1-236* mutation. Long $TG_{1\text{-}3}$ tails are detected on yeast telomeres at the end of S phase (Wellinger et al. 1993b) but not in DNA from log phase cultures except in strains that are defective in their processing, as occurs in *cdc13-1* cells growing at high temperatures (Gravel et al. 1998) or *hdf1* strains that lack the Ku heterodimeric complex (Gravel et al. 1998; Polotnianka et al. 1998). Using the nondenaturing hybridization method described in Dionne and Wellinger (1996), G-tails were not detected at *pol1-236* telomeres whereas G-tails were readily detected on *cdc13-1* and *hdf1Δ* telomeres (data not shown). Thus, by the limits of this assay, a mutation that disrupted Cdc13p–Pol1p interaction did not affect G-tail structure.

The *pol1-236* mutation eliminated interaction of Pol1Np with Cdc13Np, as monitored by the two-hybrid assay (Fig. 3C). To determine if this mutation eliminated the interaction of full-length Pol1p and full-length Cdc13p, we carried out the same experiment used to detect the interaction of these proteins in wild-type cells (Fig. 2B). A protein extract was prepared from *pol1-236* cells expressing a MYC_3-tagged Cdc13p. The extract was immunoprecipitated with anti-MYC antibodies and analyzed by Western blotting using both anti-MYC and anti-

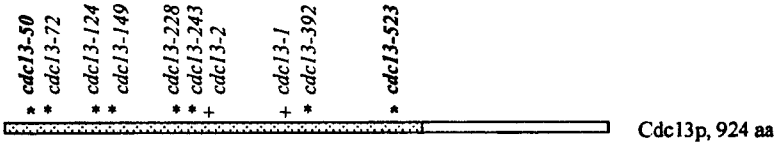
Pol1p antibodies. The amount of Pol1–236p in the immunoprecipitate was reduced substantially but not eliminated (Fig. 2B, IP panel, lane 2). The residual interaction between Cdc13p and Pol1–236p suggests that the Pol1p–Cdc13p interaction is mediated by more than one region in Pol1p and/or that other subunits of polymerase α contribute to its interaction with Cdc13p.

Mutations in CDC13 *that disrupt the Cdc13Np–Pol1Np interaction cause long telomeres*

To identify mutations in *CDC13* that disrupt the Cdc13Np–Pol1Np interaction, the 600 amino acid fragment of Cdc13p used in the initial two-hybrid screen (Fig. 1) was mutagenized by PCR. The mutagenized segments were reintroduced into the yeast two hybrid strain, and mutants that failed to interact with Pol1Np were identified by their generating white colonies in the lacZ filter assay. Of the 14 candidate mutations that produced full-length protein, DNA sequencing revealed that 12 contained a single amino acid mutation, and two contained multiple point mutations. The single mutations were spread throughout the 600 amino acids of Cdc13Np (Fig. 5A). As with the *pol1* mutations, these alleles were named according to the mutated amino acid residue. Eight of the 12 mutant polypeptides were expressed at wild-type levels, and six of these still interacted with Fun12Mp in the two hybrid assay, one of three polypeptides identified as interacting with wild-type Cdc13Np (summarized in Fig. 5B). Mutants *cdc13-50* (K50Q), *cdc13-124* (C124R), *cdc13-129* (L129S), *cdc13-228* (S228P), *cdc13-392*, (L392P) and *cdc13-523* (I523V) were defective specifically in their ability to interact with Pol1Np, and these defects were not due to reduced protein expression.

To determine the phenotypes of *cdc13* alleles that reduced the Cdc13Np–Pol1Np interaction, we introduced two of the mutations, *cdc13-50* and *cdc13-523*, on a cen-

A



B

Cdc13Np Mutation Proteins	Interaction with Pol1Np (LacZ activity)	Interaction with Fun12Mp (LacZ activity)	Protein Expression
Cdc13Np	+++	+++	+++
cdc13-50* (K50Q)	-	+++	+++
cdc13-72 (I72T)	-	-	+++
cdc13-98 (D98F)	-	+++	±
cdc13-124 (C124R)	-	+++	+++
cdc13-129 (K129R)	-	-	+
cdc13-149 (L149S)	-	+++	+++
cdc13-228 (S228P)	-	+++	+++
cdc13-243 (G243R)	-	-	+++
cdc13-382 (Y382H)	-	+++	Degraded
cdc13-392 (L392P)	-	+++	+++
cdc13-400 (V400A)	+	+++	±
cdc13-523* (I523V)	-	+++	+++
cdc13-1 (P371S)	++	NT	+++
cdc13-2 (E252K)	+++	NT	+++

C

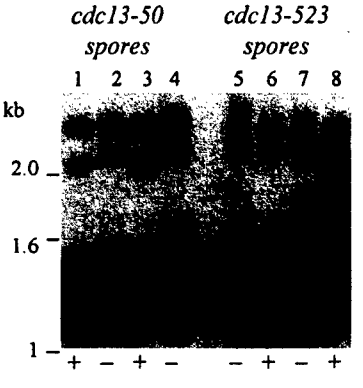


Figure 5. Alleles of *CDC13* that disrupt interactions with *POL1*. (*A*) The portion of Cdc13p that was used as bait in the two-hybrid screen is represented by the dotted region. Asterisks mark the sites of single amino acid substitutions that reduced the interaction of Cdc13Np with Pol1Np by two hybrid criteria. The alleles in bold are those used for phenotypic analyses. The locations of the *cdc13-1* and *cdc13-2* alleles are also indicated (+) (Lin and Zakian 1996; Nugent et al. 1996). (*B*) Alleles for *cdc13* polypeptides that lost interaction with Pol1Np are named by the number of the mutated residue. The amino acid change in each allele is noted in parentheses. Each mutant allele was checked for its ability to interact with both Pol1Np and Fun12Mp in the two hybrid assay using the LacZ filter assay. Western blotting was used to determine if strains carrying the mutant allele made wild-type levels of Cdc13p. (*NT*) Not tested (+++) wild-type levels of interaction or protein expression, (−) no interaction or protein expression; (±, +, and ++) intermediate levels of interaction or protein expression. (*C*) *Xho*I digested DNA from wild-type strain or *cdc13Δ* strains carrying a centromere plasmid with mutant *cdc13* alleles (*cdc13-50* and *cdc13-523*) that disrupted interaction of Cdc13Np with Pol1Np was analyzed by Southern blotting. Only the lower portion of the gels is shown. (+) Wild type (−) mutant *cdc13* alleles.

tromere plasmid into a *cdc13Δ* strain such that the mutant allele was the only copy of *CDC13* in the strain. These alleles were chosen because they produced stable protein and had little or no interaction with Pol1Np by two hybrid criteria, yet continued to interact with Fun12Mp (Fig. 5B). The growth of *cdc13-50* and *cdc13-523* cells was comparable to that of wild-type cells at 23°C, 30°C, and 37°C (data not shown), but *cdc13-50* (Fig. 5C, lanes 2,4) and *cdc13-523* (Fig. 5C, lanes 5,7) cells had telomeres that averaged 50 bp longer than wild type (Fig. 5C, lanes 1,3,6,8). As with the *pol1* alleles, this lengthening was not progressive (Fig. 4B, *cdc13-50*; cf. lanes 2 and 3). Cells carrying the *cdc13-50* and *cdc13-523* alleles had wild-type levels of TPE (Fig. 4D).

Cdc13Np interacts with Est1Cp by two-hybrid, genetic, and biochemical criteria

Telomere-bound Cdc13p has been proposed to function by recruiting telomerase to the telomere (Evans and Lundblad 1999; Zhou et al. 2000). As both Est1p (Lin and Zakian 1995; Steiner et al. 1996) and Est2p (Counter et al. 1997; Lingner et al. 1997a,b) are associated with *TLC1* telomerase RNA, we tested the ability of Cdc13Np to interact with both Est1p and Est2p in the two-hybrid assay. Full-length Est2p or the carboxy-terminal region of Est1p (Est1Cp) were cloned into the prey vector (Fig. 1). These plasmids were introduced into the two hybrid reporter strain expressing Cdc13Np. Cells expressing the Est2p fusion protein did not interact with Cdc13Np in the two-hybrid assay (Fig. 6A), although Western analysis showed that the Est2p fusion protein was expressed (data not shown). In contrast, Est1Cp, which contained the carboxy-terminal 200 amino acids of the 699 amino acid Est1p, did interact with Cdc13Np as demonstrated by the growth of cells expressing Est1Cp and Cdc13Np on plates lacking leucine (Fig. 6A,B). However, this association was weaker than the Cdc13Np–Pol1Np interaction, as expressing Cdc13Np and Est1Cp did not activate the LacZ reporter gene (data not shown). By the two hybrid assay, Est1Cp also interacted with mutant proteins Cdc13-1Np and Cdc13-2Np (Fig. 6B).

Using the biochemical approach that detected interaction of Cdc13p and Pol1p (Fig. 2B), we were unable to detect association of endogenous Cdc13p and Est1p (data not shown), perhaps because both proteins are rare and/or because their interaction within cells occurs only in a transient manner. To demonstrate the interaction biochemically, we prepared extracts from cells that overexpressed both proteins. GST–Est1p was expressed from the centromere plasmid pKT/*EST1* (Mitchell et al. 1993) using the inducible *GAL1* promoter, and HA-tagged Cdc13p was expressed from the *PGK1* promoter on the 2 μm-based plasmid pTHA/*CDC13* (Lin and Zakian 1996). Although the GST–Est1p lacked the first 31 amino acids of Est1p, it complemented an *est1* strain (Y. Yamashita, unpubl.). Extracts were incubated with glutathione sepharose (GS) beads. After extensive washing, the material bound to the beads was eluted and analyzed by Western blotting for the presence of GST–Est1p and HA–Cdc13p

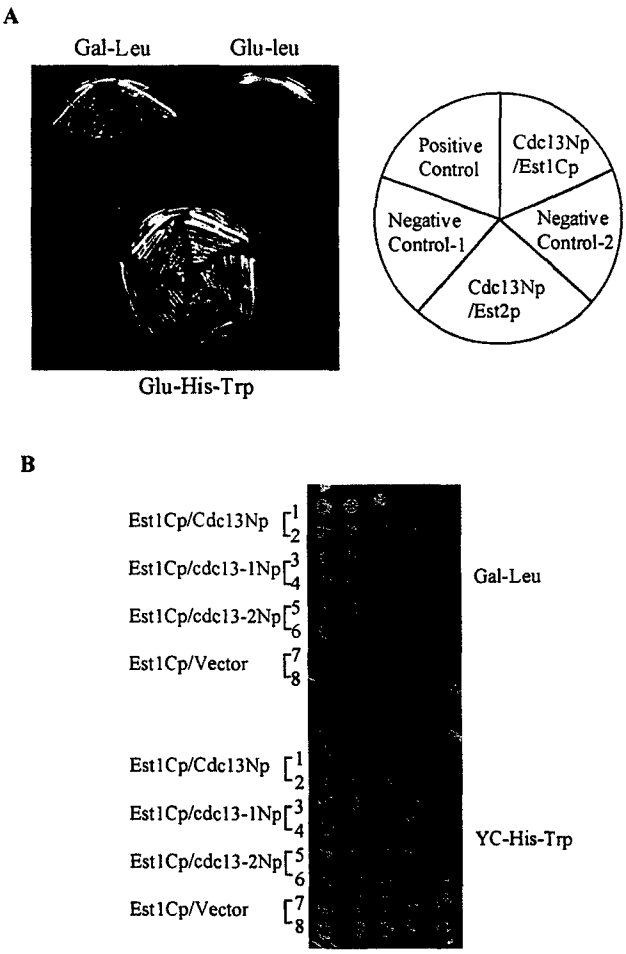


Figure 6. Cdc13Np interacts with Est1Cp in a two-hybrid assay. (*A*) Two hybrid analysis demonstrates that Cdc13Np interacted with Est1Cp but not with full-length Est2p. Positive and negative controls are the same as in Figure 2A. (*B*) Ten-fold serial dilutions of each strain were plated onto media selecting for the two-hybrid interaction (Gal - Leu) or selecting for the two plasmids (YC-His-Trp). The terminal 600 amino acids from *CDC13*, *cdc13-1*, or *cdc13-2* were tested for their ability to interact with Est1Cp.

(Fig. 7A). HA–Cdc13p was bound to the beads in extracts from cells expressing GST–Est1p (Fig. 7A, upper right panel, lane 1) but not from cells expressing just the GST protein (Fig. 7, upper right panel, lane 4) or no GST protein at all (Fig. 7, lane 6). The telomere-unrelated protein HA-eIF-5Ap did not interact with GST–Est1p (Fig. 7, lane 5). HA-Cdc13-1p (Fig. 7, lane 2) and HA-Cdc13-2p (Fig. 7, lane 3) also interacted with GST–Est1p in this assay.

To assess if the Cdc13Np–Est1Cp interaction has in vivo significance, we asked if high-level expression of Est1Cp could rescue the temperature-sensitive lethality of *cdc13-1* cells (Fig. 7B). The maximum permissive temperature for cells carrying the *cdc13-1* allele was 28°C although the isogenic wild-type strain grew well at 37°C. Ten-fold serial dilutions of wild-type or *cdc13-1* cells were spotted on galactose (gal) or glucose (glu) plates lacking tryptophan (galactose induces expression of the

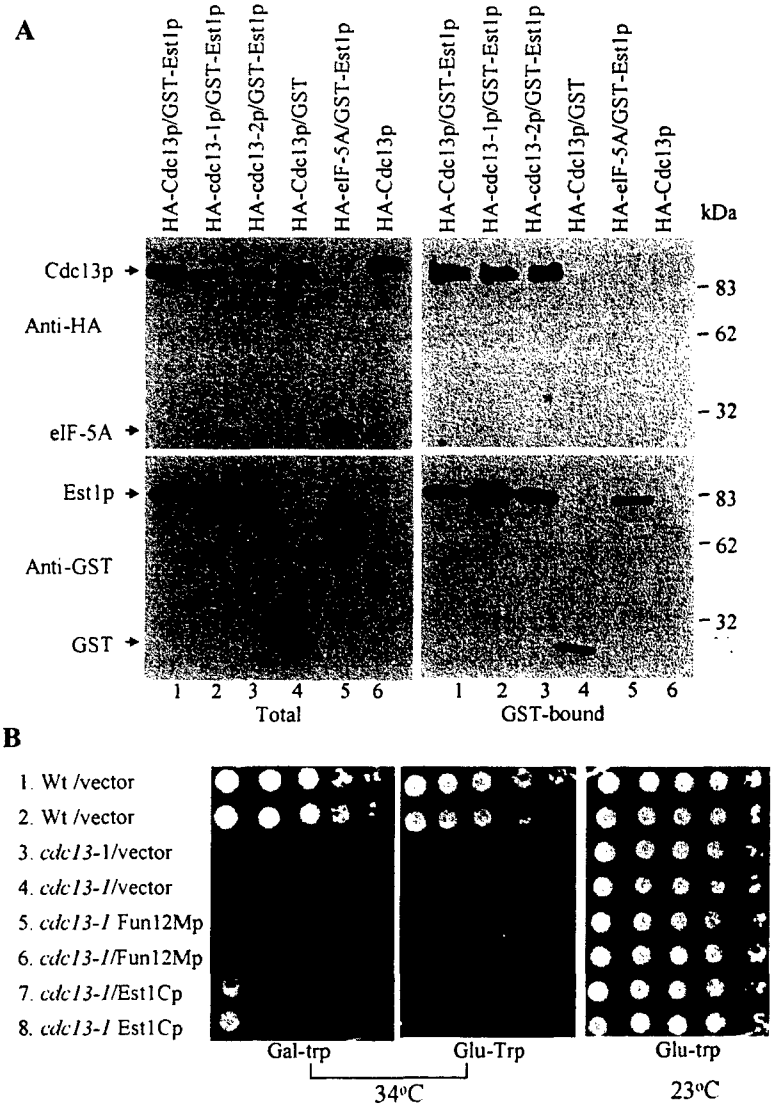


Figure 7. The interaction of Cdc13p and Est1p can be detected biochemically and has functional significance. (*A*) Extracts were prepared from cells expressing HA-tagged wild type (lanes *1,4,6*) or mutant Cdc13p (lane *2*, *cdc13-1*; lane *3*, *cdc13-2*) or HA-tagged eiF-5Ap (lane *5*). Cells also expressed GST-fused Est1p (lanes *1,2,3,5*), the GST polypeptide (lane *4*) or no GST protein (lane *6*). The total extract was analyzed by Western blotting (*left* panels) with an anti-HA (*top*) or anti-GST sera (*bottom*). The extracts were incubated with glutathione sepharose (GS) beads, and the bound proteins eluted (*right* panels) and analyzed by Western blotting as described for the unfractionated extract. (*B*) To determine the effect of Est1Cp overexpression on growth, ten-fold serial dilutions of the *CDC13* strain carrying pJG4-5 vector (lines *1,2*); *cdc13-1* carrying pJG4-5 vector (lines *3,4*); *cdc13-1* expressing Fun12Mp from the pJG4-5 vector (lines *5,6*); *cdc13-1* expressing Est1Cp from the pJG4-5 vector (lines *7,8*) were plated on galactose minus tryptophan medium and grown at 34°C (*left*) or glucose minus tryptophan medium and grown at 34°C (*middle*) or glucose minus tryptophan medium and grown at 25°C (*right*). Expression of Est1Cp and Fun12Mp was under control of the galactose inducible *GAL1* promoter.

fusion proteins; no tryptophan selects for the prey plasmid) and the plates incubated at different temperatures (Fig. 7B). The *cdc13-1* cells carried either prey vector alone, the prey vector expressing Fun12Mp, or the prey vector expressing Est1Cp. All strains grew on glucose plates at 25° (Fig. 7B, right panel); wild-type cells grew on both glucose (Fig. 7B, middle panel) and galactose (Fig. 7B, left panel) plates at 34°. However, *cdc13-1* cells did not grow at 34° unless they expressed Est1Cp: Cells with Est1Cp did not grow at 34° on glucose medium where Est1Cp was not expressed (Fig. 7B, middle panel) nor on galactose plates when expressing Fun12Mp (Fig. 7B, left panel). Thus, high-level expression specifically of Est1Cp suppressed the temperature sensitivity of *cdc13-1* cells, suggesting that the Est1Cp–Cdc13Np interaction was significant. The suppression of the *cdc13-1* growth defect was partial: *cdc13-1* cells expressing Est1Cp did not grow to the same dilution at 34° as the same strain at 25°. Also, the expression of Est1Cp did not allow *cdc13-1* cells to grow at 37° (data not shown).

Discussion

Two-hybrid analysis demonstrated that the amino-terminal two-thirds of Cdc13p interacts with the amino terminus of Pol1p, the catalytic subunit of DNA polymerase α (Fig. 2A). This interaction was confirmed by demonstrating that full-length Cdc13p and Pol1p could be coimmunoprecipitated (Fig. 2B). The interaction could be direct or indirect, mediated by one or more as yet unidentified proteins. Whether direct or not, this interaction is satisfying because polymerase α, by virtue of its primase association, is the only eukaryotic polymerase that can initiate DNA replication (Waga and Stillman 1998) and is hence the best candidate for the polymerase that resynthesizes C-strand DNA. *POL1* mutations that reduced this interaction mapped to a negatively charged, five amino acid patch within the amino-terminal 379 amino acid segment of Pol1p, identified by two-hybrid experiments (Fig. 3A). Although the catalytic subunit of DNA polymerase α is conserved

among eukaryotes, its amino-terminal portion is not conserved and no function had been mapped previously to this region (Fig. 3A).

Mutations in either *POL1* (Fig. 4A) or *CDC13* (Fig. 5C) that reduced the Pol1p–Cdc13p interaction caused telomerase-mediated telomere lengthening. For the *POL1* mutations, this *TLC1*-dependent length increase was greatest in the mutant with the most severe effect on the Pol1p–Cdc13p interaction (Figs. 3C and 4A). These genetic results argue strongly that the Pol1p–Cdc13p interaction detected by both two-hybrid (Fig. 2A) and biochemical (Fig. 2B) criteria is important for telomere maintenance. Others have shown that certain *pol1* alleles show telomerase-mediated telomere lengthening (Carson and Hartwell 1985; Adams and Holm 1996). However, in these cases the mutations are temperature sensitive, affect the catalytic activity of Pol1p, and have global effects on both DNA replication and cell viability. In contrast, the *pol1* mutations studied here occurred in a region that is thought to be dispensable for catalysis, and these mutations had no effect on cell growth or TPE (Fig. 4D). Because these mutations disrupted interaction with Cdc13p (Figs. 2B and 3C), an in vivo telomere binding protein (Bourns et al. 1998), their effects on telomere length were likely direct. Based on these data, we propose that telomere-bound Cdc13p recruits polymerase α to the telomere and that this interaction promotes C-strand resynthesis.

If the Pol1p–Cdc13p interaction is essential for C-strand resynthesis, mutations like *pol1-236* that eliminated this interaction by two-hybrid criteria (Fig. 3C) should be lethal due to loss of telomeric DNA and/or activation of DNA damage checkpoints by long ss G-tails (Lee et al. 1998). However, biochemical experiments demonstrated that there is residual interaction between Cdc13p and Pol1p even in the *pol1-236* strain (Fig. 2B), suggesting that other parts of Pol1p might also interact with Cdc13p. Alternatively or in addition, other telomere proteins (such as Stn1p) whose loss has a similar effect on C-strand degradation as the *cdc13-1* mutation (Grandin et al. 1997), or the Ku heterodimer, whose loss results in G-tails throughout the cell cycle (Gravel et al. 1998; Polotnianka et al. 1998), might be partially redundant with Cdc13p and help recruit Pol1p to the telomere.

We also report that the amino terminus of Cdc13p interacted with the carboxyl third of Est1p by two-hybrid analysis (Fig. 6A). Biochemical experiments confirmed this interaction, demonstrating that full-length Cdc13p interacted in vivo with a close to full-length, functional Est1p (Fig. 7A). Again this interaction could be direct or mediated by another protein. As overexpression of the carboxy- terminal 200 amino acid segment of Est1p partially suppressed the temperature sensitive lethality of *cdc13-1* cells whereas overexpression of another Cdc13p interacting polypeptide, Fun12Mp, did not (Fig. 7B), the Cdc13p–Est1p interaction is likely to be important for the essential function of Cdc13p.

Because Est1p is associated with telomerase RNA (Lin and Zakian 1995; Steiner et al. 1996), a Cdc13p–Est1p interaction could recruit telomerase to telomeres, thereby explaining the senescence phenotype of *cdc13-2* cells. That Cdc13p functions to recruit telomerase is also supported strongly by the demonstration that expression of a Cdc13p–Est1p or a Cdc13p–Est2p fusion protein supplants the need for Est1p in telomere maintenance (Evans and Lundblad 1999). However, as these fusion proteins might bypass the normal role of Est1p, these data do not address whether Cdc13p and Est1p interact in vivo, whereas our data provide strong support for this possibility.

If Cdc13p recruits telomerase by virtue of its ability to interact with Est1p, the phenotype of *cdc13-2* cells could be explained if Cdc13-2p were unable to interact with Est1p. However, Est1p interacted with Cdc13-2p as assayed by both two-hybrid (Fig. 6B) and biochemical (Fig. 7A) criteria. One explanation for this result is trivial: Est1p might be able to interact with Cdc13-2p when both proteins are overproduced as they were in both the two-hybrid and biochemical assays, but not when the proteins are expressed at physiological levels. This possibility predicts that overexpression of Est1p would suppress the telomere defects of a *cdc13-2* strain. However, overexpression of a GST–EST1p fusion protein that contained all but the most amino-terminal 31 amino acids of Est1p, and which complemented an *est1* strain or overexpression of the carboxy-terminal portion of Est1p, did not suppress the telomere length defect of *cdc13-2* cells (data not shown). Alternatively, interaction of Cdc13p with Est1p might trigger a conformational change in Cdc13p, such as phosphorylation, that makes it better able to promote the telomerase pathway: Cdc13-2p might be able to interact with Est1p but not undergo the subsequent conformational change.

In the ciliate *Euplotes*, aphidicolin, an inhibitor of conventional DNA polymerases, causes changes in the length of both the C- and G-strands of telomeric DNA. This result led to the proposal that G-strand lengthening and C-strand synthesis are coordinately regulated (Fan and Price 1997). In *Saccharomyces*, addition of duplex telomeric DNA to a double-strand break requires not only telomerase but also DNA primase and two of the three conventional DNA polymerases, polymerase α and polymerase β (Diede and Gottschling 1999). These data also support a model of coordinate regulation of C- and G-strand synthesis. Cdc13p, by virtue of its ability to interact with both the conventional replication apparatus (Fig. 2A) and a telomerase component (Fig. 6A), could play a critical role in this coordination.

Alternatively, there might be competition between C-strand resynthesis by polymerase α and G-strand lengthening by telomerase. In support of this model, when the interaction between Cdc13p and Pol1p was weakened, telomerase-mediated lengthening of telomeres increased (Fig. 4A). By two hybrid criteria, Cdc13p interacted more strongly with Pol1p than it did with Est1p. Also, polymerase α is much more abundant than telomerase. Based on these considerations, a competition model predicts that recruitment by Cdc13p of polymerase α to ss TG_{1-3}

tails is much more likely than recruitment of telomerase. As short telomeres are preferentially lengthened by telomerase (Marcand et al. 1999), telomere length may influence this competition.

Materials and methods

The yeast two-hybrid strain YEM1α (*MATa his3-11 trp1-1 LEU2::pLexAop-LEU2 ura3-1:: URA3-pLexAop-Gal1-lacZ*) was constructed by E. Monson in our lab using reagents described in Gyuris et al. (1993). The protease-deficient yeast strain BJ2168 (Jones 1991) was used to detect Est1p–Cdc13p interaction. YPH499, YPH500, and YPH501 (Sikorski and Hieter 1989) were used for analysis of *pol1* and *cdc13* mutant phenotypes. *CDC13* deletion in YPH501 was created by J.J. Lin (Lin and Zakian 1996). Cdc13p was tagged at its carboxyl terminus with three MYC epitopes (Schneider et al. 1995) in strain VPS106 (V. Schulz and V. Zakian 1994) and used for immunoprecipitation. *CDC13* was also tagged with nine MYC epitopes at its carboxyl terminus in strain YPH499 (A. Taggart and V.A. Zakian, unpubl.) as in Zachariae et al. (1996). Cells containing either 3-myc-tagged or 9-myc-tagged *CDC13* had normal cell growth and telomere length.

The two-hybrid assay was done essentially as described (Gyuris et al. 1993) using components generously provided by Dr. R. Brent and colleagues. The prey library (pJG4-5/X) (Gyuris et al. 1993; Golemis et al. 1994) was transformed into YEM1α strain containing the *CDC13N* bait plasmid (pEG202/*CDC13*-B; constructed by J.-J. Lin) (Fig. 1). HA_3-tagged *CDC13* from pTHA/*CDC13* (Lin and Zakian 1996) was inserted into *Bam*HI–*Sal*I digested pVZ1, then *Bgl*II digested, followed by self-ligation to delete the *Bgl*II fragment within *CDC13*. This introduces a frameshift at the ligation site such that only the first 600 amino acids of Cdc13p are produced. The HA-tagged amino-terminal region of *CDC13* was cloned into pEG202 generating a LexA DNA binding domain–Cdc13Np fusion protein expressed from the *ADH1* promoter. The library proteins were also HA tagged and expressed from a *GAL1* promoter. Transformants were harvested and replated to 3% galactose YC plates minus leucine. Leu^+ colonies were transferred to nitrocellulose filters (Schleicher & Schuell) for the colony *lacZ* filter assay (Breeden and Nasmyth 1985). Liquid lacZ assays were done as in Miller (1972) and Guarente (1983). Leu^+ $LacZ^+$ colonies were purified and confirmed by rescuing the plasmids into *E. coli* and retransforming them into the two-hybrid yeast strain. Prey plasmids that retested positively were sequenced with the amplitaq FS dye terminator cycle sequencing kit (ABI).

The plasmid pJG4-5/*EST1C* was constructed by Y. Yamashita and contained amino acids 499–699 of Est1p fused in-frame with the B42 trans-activation domain and an HA epitope tag in the prey vector pJG4-5. Full-length *EST2* was PCR amplified from YPH499 genomic DNA and cloned into pJG4-5 to create pJG4-5/*EST2* (made by S-C. Teng). This plasmid complements an *est2Δ* strain. The *cdc13-1* mutation P371S creates an additional *EcoR*I site (Lin and Zakian 1996) and *cdc13-2* mutation E252K eliminates the first *EcoR*I site in *CDC13* (Nugent et al. 1996). pEG202/*cdc13-1B* and pTHA/*cdc13-1* were created by site-directed mutagenesis (QuikChange Site-Directed Mutagenesis Kit, Stratagene) from pEG202/CDC13N and pTHA/CDC13, respectively. pEG202/*cdc13-2B* and pTHA/*cdc13-2* were created from pEG202/CDC13N and pTHA/CDC13, respectively. The mutated plasmids were introduced into *E. coli*, purified, and then sequenced to confirm that the correctmutation was made. Plasmids producing LexA::cRafp or LexA::Krev1p fusion protein were gifts from E. Golemis.

Immunoprecipitation to detect Cdc13p–Pol1p interaction was done in yeast strain VPS106 containing 3XMYC-*CDC13* as in Garrett et al. (1991), except that cells were lysed in a homogenizer (Avestin's High Pressure Homogenizer EmulsiFlexC-5) in 40 mM Tris (pH 8.0), 75 mM NaCl, and 20 mM KOAc, 1 mM 2-mercaptoethanol, 0.01% NP-40, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml pepstain A, 5 μg/ml leupeptin, 10 μg/ml aprotinin, 10 mM benzamidine, 250 μg/ml soybean trypsin inhibitor, and 10% glycerol. Monoclonal anti-MYC 9E10 (Princeton University) was used for the immunoprecipitation. Monoclonal anti-Pol1p antibody (from P. Plevani), anti-MYC antibody, and ECL (Amersham Life Science) were used to visualize the proteins.

The GST (glutathione S transferase) assay was conducted essentially as described in Mitchell et al. (1993). pEGKT/*EST1*, a plasmid that produces a GST::Est1p fusion protein under the control of a *GAL1* promoter, was constructed and generously provided by Y. Yamashita. In brief, the *Hinc*II fragment from *EST1*, which contains amino acids 31–699 of Est1p, was cloned into pEGKT (Mitchell et al. 1993). Expression of GST::Est1p from this plasmid complements an *est1Δ* mutant (data not shown). Full-length Cdc13p tagged with 3 × HA at its amino terminus was expressed from a *PGK1* promoter on plasmid pTHA/*CDC13* (Lin and Zakian 1996). The two proteins were coexpressed in strain BJ2168. A similarly HA-tagged version of the yeast protein eIF-5A (Kang and Hershey 1994) was constructed by J-J. Lin and used as a negative control. Raffinose-grown log phase cells were transferred to 3% galactose medium and incubated at room temperature overnight to induce expression of *GAL1*-regulated genes. Cells were lysed in 20 mM Tris (pH 8.0), 200 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.01% NP-40, 10% glycerol with protease inhibitors plus an equal volume of glass beads by vortexing in mini-beadbeater-8 (Biospec Products). NP-40 and Triton X-100 were added to the soluble protein fractions to final concentrations of 0.5% and 0.1%, respectively. GST-Sepharose 4B beads (Pharmacia Biotech) were then added. After incubation at 4°C, beads were collected by centrifugation and then washed once with the lysis buffer containing 1% NP-40 0.1% Triton X-100 and once with lysis buffer containing 450 mM NaCl. The beads were treated with 0.1 mg/ml DNase I in lysis buffer plus 1 mM $MgCl_2$, then washed with lysis buffer containing 450 mM NaCl and 350 mM KOAC. Proteins were visualized by ECL Western using anti-HA antibody 12CA5 (Princeton University) and rabbit anti-GST antibodies (from Jin-Qiu Zhou).

PCR mutagenesis was used to generate *POL1* and *CDC13* mutations (Grandin et al. 1997). Two DNA oligonucleotides that were complementary to the sequences from the prey vector pJG4-5 (for *POL1* mutations) or to the bait vector pEG202 (for *CDC13*) were used. A modified random PCR mutagenesis (Leung et al. 1989; Zhou et al. 1991) was done in a reaction mixture containing 100 μM dNTP, 200 νM oligonucleotides, 1× PCR buffer with 3 mM $MgCl_2$, 1–10 ng/100 μl plasmid DNA template, and 1 unit Taq polymerase (Roche Molecular Biochemicals). PCR products were cotransformed into the two-hybrid strain containing bait or prey plasmids with *Xho*I linearized pJG4-5 or pEG202 vector. Pools of the PCR mutagenized *POL1* segments were introduced by transformation into the yeast strain containing both the two-hybrid reporter genes and expressing Cdc13Np (Grandin et al. 1997). Transformants were selected by their ability to grow on media lacking histidine and tryptophan, thus selecting for both the prey and bait plasmids but not for the two-hybrid interaction. Transformants were replica plated to galactose plates then transferred to nitrocellulose membranes for LacZ assays.

The *pol1-236*, *pol1-238*, and *pol1-241* mutant alleles were in-

troduced into the *POL1* integration plasmid pCM1 (Lucchini et al. 1988) using the QuickChange site-directed Mutagenesis Kit (Stratagene). Silent mutations that created an *AclI* site in *pol1-236* (T_{708}→C and G_{710}→T in addition to the D236N mutation, G_{706}→A) or an additional *EcoRI* site in *pol1-238* and *241* (A_{713}→G in addition to the E238K mutation, G_{711}→A or P241T, C_{720}→A) were also introduced to allow identification of mutant alleles by restriction enzyme digestion. The mutagenized plasmids were linearized by *Bst*XI digestion and integrated into the *POL1* locus of diploid strain YPH501 as in Lucchini et al. (1988). Transformants were selected by their Ura$^+$ phenotype and verified by Southern. The diploid cells were sporulated; spores containing the mutant *POL1* alleles were identified by Ura$^+$ phenotype. Cells that excised *URA3*, leaving behind an intact but mutated *POL1* gene, were selected on plates containing 5-fluoro orotic acid.

Wild-type *CDC13* with its own promoter and terminator sequences was released from YEP24/*CDC13* using *ApaI* digestion (Garvik et al. 1995) and cloned into the *ApaI*-digested *CEN* plasmid pRS314. The pRS314 /*CDC13* was mutagenized using the QuickChange mutagenesis kit. To facilitate identification of mutations, an *AvrII* site was created near K50Q in pRS/*cdc13-50* and the first *HindIII* site of CDC13 was eliminated at I523V in pRS314/*cdc13-523* without changing additional amino acid residues. The mutated plasmids were introduced into a derivative of YPH 501 that had one copy of *CDC13* and one copy of *cdc13::HIS3* (Lin and Zakian 1996). After sporulation and dissection, haploid *cdc13::HIS3* spores carrying the mutated *cdc13* plasmid were selected by their His$^+$ Trp$^+$ phenotype.

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