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Evidence Suggesting that Pif1 Helicase Functions in DNA Replication with the Dna2 Helicase/Nuclease and DNA Polymerase δ

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The precise machineries required for two aspects of eukaryotic DNA replication, Okazaki fragment processing (OFP) and telomere maintenance, are poorly understood. In this work, we present evidence that *Saccharomyces cerevisiae* Pif1 helicase plays a wider role in DNA replication than previously appreciated and that it likely functions in conjunction with Dna2 helicase/nuclease as a component of the OFP machinery. In addition, we show that Dna2, which is known to associate with telomeres in a cell-cycle-specific manner, may be a new component of the telomere replication apparatus. Specifically, we show that deletion of *PIF1* suppresses the lethality of a *DNA2*-null mutant. The *pif1*Δ *dna2*Δ strain remains methylmethane sulfonate sensitive and temperature sensitive; however, these phenotypes can be suppressed by further deletion of a subunit of pol δ, *POL32*. Deletion of *PIF1* also suppresses the cold-sensitive lethality and hydroxyurea sensitivity of the *pol32*Δ strain. Dna2 is thought to function by cleaving long flaps that arise during OFP due to excessive strand displacement by pol δ and/or by an as yet unidentified helicase. Thus, suppression of *dna2*Δ can be rationalized if deletion of *POL32* and/or *PIF1* results in a reduction in long flaps that require Dna2 for processing. We further show that deletion of *DNA2* suppresses the long-telomere phenotype and the high rate of formation of gross chromosomal rearrangements in *pif1*Δ mutants, suggesting a role for Dna2 in telomere elongation in the absence of Pif1.

Yeast Pif1 is the founding member of the Pif1 subfamily of superfamily 1 DNA helicases (3). While other organisms, such as *Caenorhabditis elegans* and *Homo sapiens*, have only one identified Pif1 family member, in yeast, there is a second, closely related, protein, Rrm3p (3). In yeast, neither of these helicases is essential and mutants lacking both are viable and repair proficient. Both yeast proteins have 5′-to-3′ DNA helicase activity (21, 30, 31). The region of similarity between Pif1 and Rrm3 is limited to the seven helicase motifs, which exhibit 40% identity and 60% similarity (3). This may indicate that the two helicases have structurally similar DNA substrates. Nevertheless, the two helicases differ in their biological functions, and these differences are likely mediated not only by the helicase domain but also by the divergent N termini, which are not required for helicase activity (4). To date, it has been impossible to determine which helicase, Rrm3 or Pif1, is the functional homolog of the single ortholog in other eukaryotes.

One difference between Rrm3 and Pif1 is in their function at the rRNA gene. In *rrm3* mutants, there is an increase in replisome pausing at the Fob1 protein-bound replication fork barrier (RFB) in the rRNA gene (22). The hypothesis is that Rrm3 is required to remove proteins that block the fork at that point, since Rrm3 is required for promoting fork movement at over 1,400 loci in the yeast genome, in addition to the RFB. In contrast to Rrm3, Pif1 seems to be required for pausing at the rRNA gene RFB, since *pif1*Δ mutants show a threefold-reduced number of forks accumulating at the RFB. Pif1 could itself be an inhibitor of fork movement, or it could be required for Fob1p to exert its blocking action (22, 50).

Pif1 and Rrm3 also appear to have different roles at telomeres. Two-dimensional gel analysis of telomere replication in *rrm3*Δ mutants suggests that Rrm3 is required for replication through Y′ elements and telomeric repeats, and similar experiments in *rrm3*Δ *rap1* strains further suggest that Rrm3 specifically removes Rap1 protein, or proteins that associate with telomeres in the absence of Rap1, to allow progression of the telomere-proximal replication fork (21, 35). Pif1, on the other hand, is thought to be an inhibitor of telomerase, since *pif1*Δ mutants have long telomeres, 160 to 240 bp longer than normal telomeres, because overproduction of Pif1 shortens telomeres and since Pif1 decreases the processivity of telomerase in vitro (5). *pif1*Δ mutants also show a 200-fold increase in the de novo addition of telomeres at HO-endonuclease-induced double-strand breaks (DSBs) and a high level of gross chromosomal rearrangements (GCRs) in which there is excessive telomere addition to nontelomeric sequences at sites of intrachromosomal DNA damage, presumably primarily DSBs (36, 42, 50). By analogy with Rrm3, it has been proposed that Pif1 might remove a protein such as telomerase from the chromosome ends. *pif1*Δ mutants also have decreased silencing in the subtelomeric repeats.

A third possible difference between Pif1 and Rrm3 is that Pif1, but not Rrm3, is required for mitochondrial DNA recombination and genome maintenance (30, 31, 42). The *pif1*Δ mutant grows slowly on nonfermentable carbon sources and loses functional mitochondria rapidly (42). *RRM3* does contain a mitochondrial import signal; however, it has not yet been shown to function in mitochondria (1, 3, 18, 23).

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TABLE 1. Strains used in this study

Strain	Characteristics	Source or reference
BY4741	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Invitrogen
BY4742	*MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Invitrogen
W303	*MAT***a** *trp1-1 ura3-1 his3-11,15 leu2-3,112 ade2-1 can1-100 RAD5*	H. Klein
4741dna2-1-6D	*MAT***a** *dna2-1 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	This study
4741dna2-2-11D	*MATα dna2-2::LEU2 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	This study
MB110	*MAT***a** *dna2Δ::kanMX his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 trp1Δ* (pSEY18GALDNA2)	
MB201	BY4741 *MAT***a** *pif1Δ::HIS3 trp1Δ*	This study
MB202	BY4742 *MAT***a** *pif1Δ::HIS3 trp1Δ*	This study
10509	BY4742 *MATα pif1Δ::kanMX*	This study
MB203.3	BY4741 *MAT***a** *dna2Δ::kanMX pif1Δ::HIS3 trp1Δ*	This study
MB203.5	BY4741 *MAT***a** *dna2Δ::kanMX pif1Δ::HIS3 trp1Δ*	This study
MB203.6	BY4741 *MAT***a** *dna2Δ::kanMX pif1Δ::HIS3 trp1Δ*	This study
MB204	BY4742 *MATα dna2Δ::kanMX pif1Δ::HIS3 trp1Δ*	This study
MB205	BY4741 *MAT***a** *pol32Δ::natR trp1Δ*	This study
MB206	BY4742 *MATα pol32Δ::natR trp1Δ pif1Δ::HIS3*	This study
MB207	BY4741 *dna2Δ::kanMX pif1Δ::HIS3 pol32Δ::natR trp1Δ*	This study
MB208	BY4741 *MATα dna2-2 pif1Δ::kanMX*	This study
579-6D	*MAT***a** *trp1-1 ura3-1 his3-11,15 leu2-3,112 ade2-1 can1-100*	H. Klein
580-10D	*MAT***a** *trp1-1 ura3-1 his3-11,15 leu2-3,112 ade2-1 can1-100*	H. Klein
3615	*MAT***a** *ura3-52 leu2Δ1 trp1Δ63 his3Δ200 lys2* Bgl *hom3-10 ade2-Δ1 ade8 hxt13::URA3*	36
4400	*MAT***a** *pif1Δ::kanMX ura3-52 leu2Δ1 trp1Δ63 his3Δ200 lys2* Bgl *hom3-10 ade2-Δ1 ade8 hxt13::URA3*	36
4393	*MAT***a** *pif1-m1 ura3-52 leu2Δ1 trp1Δ63 his3Δ200 lys2* Bgl *hom3-10 ade2-Δ1 ade8 hxt13::URA3*	36
4344	*MAT***a** *pif1-m2 ura3-52 leu2Δ1 trp1Δ63 his3Δ200 lys2* Bgl *hom3-10 ade2-Δ1 ade8 hxt13::URA3*	36
4344dna2Δ	4344 *dna2Δ::HIS3*	This study
3615-2-1	3615 *dna2-1::natR*	This study
3615-2-2	3615 *dna2-2::natR*	This study
3615-K1080A	3615 *dna2-K1080A::natR*	This study
5030	*MAT***a** *pif1-m2 ura3-52 leu2Δ1 trp1Δ63 his3Δ200 lys2* Bgl *hom3-10 ade2-Δ1 ade8 hxt13::URA3*	36, 49
5030dna2Δ	5030 *dna2Δ::HIS3*	
509	*MAT***a** *pif1Δ::kanMX his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Invitrogen
MB90-7A	*MAT***a** *dna2-1 trp1-1 ura3-1 his3-11,15 leu2-3,112 ade2-1 can1-100*	This study
MB91	*MAT***a** *dna2-1pif1Δ::HIS5 trp1-1 ura3-1 his3-11,15 leu2-3,112 ade2-1 can1-100*	This study
U953-61A	*MAT***a** *mec1Δ::TRP1 sml1::HIS trp1-1 ura3-1 his3-11,15 leu2-3,112 ade2-1 can1-100*	49
MB92-M	*MAT***a** *dna2-1 mec1Δ::TRP1 sml1::HIS3 trp1-1 ura3-1 his3-11,15 leu2-3,112 ade2-1 can1-100*	11
SPY40	*MAT***a** *tel1::URA3 trp1-1 ura3-1 his3-11,15 leu2-3,112 ade2-1 can1-100*	40
MB92-31C	*MAT***a** *dna2-2 mec1Δ::TRP1 sml1Δ::HIS3 trp1-1 ura3-1 his3-11,15 leu2-3,112 ade2-1 can1-100*	11
MB92-35A	*MATα dna2-2 tel1Δ::URA3 sml1Δ::HIS3 trp1-1 ura3-1 his3-11,15 leu2-3,112 ade2-1 can1-100*	11
U960-5C	*MAT***a** *trp1-1 ura3-1 his3-11,15 leu2-3,112 ade2-1 can1-100 rad53::HIS3 sml1-1*	49
MB92-1C	*MAT***a** *dna2-1 tel1Δ::URA3 sml1Δ::HIS3 trp1-1 ura3-1 his3-11,15 leu2-3,112 ade2-1 can1-100*	11
MB504	*MAT***a**/α *PIF/pif1Δ::HIS3 DNA2/dna2Δ::kanMX CDC13/cdc13-1*	This study

In this work, we report an additional difference between Pif1 and Rrm3. The *rrm3Δ* mutant is synthetically lethal with a mutation in the Dna2 helicase/nuclease, required for Okazaki fragment processing (OFP), telomere stability, and DNA repair (6–10, 14, 17, 47, 48). In contrast, we show here that *pif1Δ* suppresses both the DNA replication and repair defects of *dna2* mutants and even the lethality of deletion of *DNA2*. The *dna2Δ pif1Δ* mutant retains some defects in replication and repair, but these are further suppressed by deletion of the *POL32* subunit of polymerase δ (pol δ). Conversely, deletion of *DNA2* suppresses the telomere phenotype of *pif1Δ* mutants. We describe and characterize here genetic interactions between *PIF1*, *DNA2*, and *POL32* that present new insights into the functions of each gene in OFP, telomere function, and chromatin modification and into the evolutionarily conserved functions of Pif1 helicase.

MATERIALS AND METHODS

Strains. The strains used in this study were derived either from BY4741, in which the yeast deletion collection is housed (Invitrogen, Carlsbad, Calif.), or from W303 RAD5^{+}. Individual experiments compared only isogenic strains in either background. The full strain list is shown in Table 1. Standard genetic techniques were used for tetrad analysis. Gene disruptions were carried out by standard PCR-based methods and verified by PCR. Sequences of primers not shown below are available on request.

Oligonucleotides. The following oligonucleotides were used for gene disruption by standard genetic techniques (34): dna2NΔ (CAAGTGAGTACTCATTTTGTGCAAGCAAACACTGACAATTGAAGAGATCGTCAGGCGGATCCCCGGGTTAATTAA), dna2CΔ (TATTTTATGCTGTGATAGCTTTCCTGTTATGGAGAAGCTCTTCTTATTCCCCCTGGAATTCGAGCTCGTTTAAAC), dna2Nseq (CAATAAAGCAATTCCGTGCGGCAGA), pif1NΔ (ATTTTGATATATTATCCATTGAGCGATTAGCTTACTTGTATCAATCAATTTTACCGGATCCCCGGGTTAATTAA), pif1CΔ (GATTATTATAGCAGTTTGTATTCTATATAACTATGTGTATTAATATGTTACGAATTCGAGCTCGTTTAAAC), pTEF kan (CTCGCAGGTCTGCAGCGAGGAGCCG), pif1-95 (GGCCAGACATTGAAACTGG), and dna2-seq (CAATAATGCAATTCCGTGCGGCAGA).

Verification of deletions of *PIF1* or *DNA2*. We verified our earlier results and those of others that *DNA2* is essential (6, 8, 17, 43). For the studies reported here, a heterozygous *dna2Δ* strain was obtained from Research Genetics as clone 22858, and dissection after sporulation shows that in the strain, *DNA2* is essential. Complementation with a plasmid containing a functional *DNA2* gene rescues the deletion, and transformation with constructs containing nonfunctional *dna2* genes, *dna2K1080E*, d*na2E675A*, *dna2E657A*, and *dna2E693A*, does not rescue the deletion (10; unpublished data). Therefore, the *dna2Δ pif1Δ* mutant derived from a haploid segregant of clone 22858 is unlikely to contain any

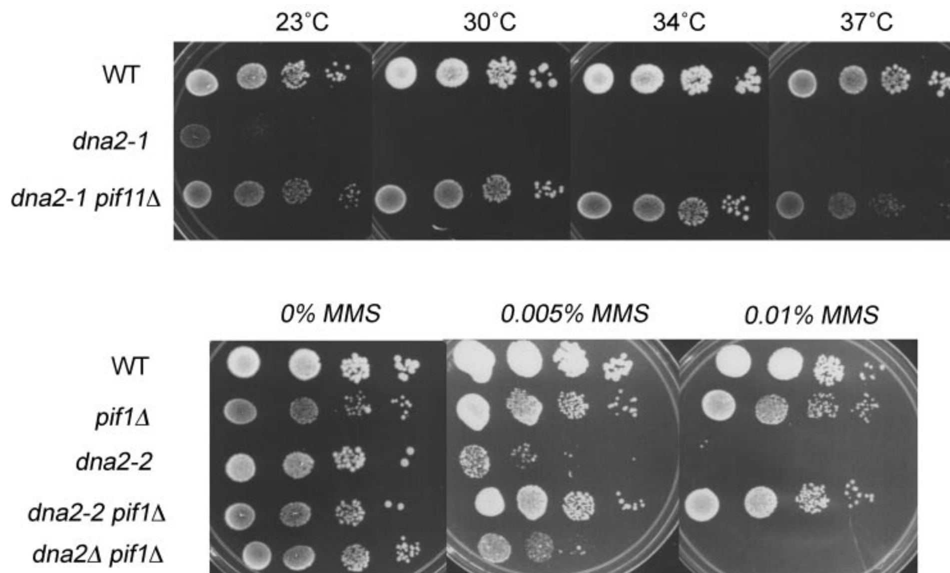

FIG. 1. Suppression of *dna2* mutant phenotypes by deletion of *PIF1*. (A) Serial dilutions of identical numbers of cells were carried out at the temperatures indicated. The following strains were used: W303, WT; MB90-7A, *dna2-1*; MB91, *dna2-1 pif1*Δ. (B) Serial dilutions of identical numbers of each strain were plated on medium containing the indicated amounts of MMS. Relevant genotypes are indicated. The following strains were used: BY4741, WT; 10509, *pif1*Δ; 4741dna2-2, *dna2-2*; MB208, *dna2-2 pif1*Δ; and MB203, *dna2*Δ *pif1*Δ (Table 1).

aberrant mutations, rearrangements, or unknown suppressors which are linked to *DNA2*. The *DNA2* gene is also essential in *Schizosaccharomyces pombe* (26). All constructs containing deletions of these genes were checked in multiple ways. For physical analysis of strains used in Fig. 1B, colony PCR was used, permitting application to large numbers of transformants in a single experiment. The oligonucleotides used in PCR are available on request. The *pif1*Δ strain was confirmed using oligonucleotides pTEF kan and pif1-95. The resulting PCR product was 350 bp. The *dna2*Δ strains were confirmed using the oligonucleotides pTEF kan and dna2-seq. The resulting PCR product was 450 bp. All strains used in the subsequent experiments presented were confirmed in this way.

For verification of strains used in generating and in analyzing the genotype of strain MB504 (the double heterozygote used in the Results section to test lethality of *dna2*Δ and its suppression by deletion of *PIF1*), colony PCR was used; the primers are available on request.

Five strains were obtained from Richard Kolodner: 3615 (*PIF1*), 4393 (*pifm1*), 4344 (*pifm2*), 5030 (*pif1m2*), and 4400 (*pif1*Δ). The PCR product used to delete the *DNA2* gene contained the *HIS3* gene flanked by 55 bp of DNA homologous to *DNA2* on the N- and C-terminal regions. The His^+ transformants were picked and tested for temperature sensitivity, growth on glycerol, and methylmethane sulfonate (MMS) sensitivity. The temperature-sensitive (recessive, ts; dominant, TS) transformants were further tested by either colony PCR for the *dna2*Δ::*HIS3* deletion or complementation using a *dna2* strain. Strain 4400 (*pif1*Δ) had 26 transformants: 7 ts and 19 TS^+. All ts transformants were *dna2*Δ. 4344 (*pif1m2*) had 31 transformants: 20 ts and 11 TS^+. All ts transformants were *dna2*Δ. 5030 (*pif1m2*) had 32 transformants: 24 ts and 8 TS^+. All ts transformants were *dna2*Δ. 4393 (*pif1m1*) had 32 transformants: 0 ts and 32 TS^+. 3615 had 12 transformants: 0 ts and 12 TS^+. None of these were *dna2*Δ. The PCR product is quite efficient in deleting the Dna2 gene in the *pif1*Δ or *pif1m2* background, but not the $PIF1^+$ or *pif1m1* background. *dna2*Δ *pif1-m2* transformants were also checked by complementation. The *dna2*Δ::G418 *pif1-m2* strain was mated to a *dna2-1 LEU2* strain, and $G418^R$ Leu^+ diploids were selected. A *dna2-1*/*dna2*Δ *PIF1*/*pif1-m2* strain fails to grow at 37°C, while a *dna2-1*/*DNA2 PIF1*/*pif1-m2* diploid does grow at 37°C.

Additional phenotypic analysis of mutant constructs is described in the text where appropriate.

Determination of telomere length. Chromosomal DNA was cleaved with XhoI, loaded onto a 1% agarose gel, and electrophoresed for 6 h at 60 V. The gel was blotted onto Gene Screen Plus and hybridized using a 300-bp double-stranded GT probe as described previously (14). Since telomere length is clonal, three independent isolates were analyzed for each strain. The hybridization "smear" at about 1.3 kb represents the terminal fragment of Y′ telomeres cut by XhoI and is indicative of telomere length in each strain.

RESULTS

Suppression of *dna2-1* by *pif1*Δ. We have reported that *dna2-2* is synthetically lethal with *rrm3*Δ (47). *RRM3* is highly homologous to *PIF1*, and they affect to some extent similar regions of the genome. To further understand the *rrm3*Δ *dna2-2* synthetic lethality, we wished to examine *dna2-2 pif1*Δ double mutant strains. *dna2-2* mutants (R1235Q) have a mutation in the helicase domain and are expected to have a substantial defect only in the helicase activity, although the dna2-2 protein has never been directly studied (17). When we constructed a *dna2-2 pif*Δ double mutant, instead of the expected synthetic lethality, we found that *dna2-2 pif1*Δ double mutants were viable. *dna2-2* cells are viable but grow slowly, and when grown on plates most of the cells are budded, often with large buds, consistent with a previously established G_2/M delay (17; unpublished observations). We noticed that *dna2-2 pif1*Δ had the same morphology as wild-type (WT) cells, small and unbudded (not shown), suggesting that *pif1*Δ might suppress the *dna2-2* G_2/M delay.

To further test the proposed suppression, we investigated the effect of deletion of *PIF1* on a *dna2* mutant with a more defined phenotype, the *dna2-1* strain. *dna2-1* is a temperature-sensitive allele (P504S) that maps in the nuclease domain of *DNA2* and drastically reduces the nuclease activity of the Dna2 protein (10). Given that *pif1*Δ reduces pausing at the rRNA gene RFB, we expected *pif1*Δ mutants might show some suppression of *dna2-1*, since *fob1*Δ, which also decreases pausing at the RFB, suppresses the DNA damage sensitivity in *dna2* mutants (47, 48). The *PIF1* gene was deleted by PCR-mediated mutagenesis in a W303 *dna2-1* strain, which was confirmed as described in Materials and Methods. As expected, deletion of *PIF1* suppressed the growth defect of *dna2-1* strains at 23°C and the lethality of *dna2-1* strains at 34°C (Fig. 1A). Unexpect-

edly, the *dna2-1 pif1Δ* strain was even viable at 37°C, although the wild-type growth rate was not completely restored at 37°C (Fig. 1A). (The *dna2-1* strain grows slowly even at 23°C, and plates at 23°C were photographed before they were fully grown so that the other strains would not be overgrown, accounting for the appearance of Fig. 1A. Further incubation verified that the *dna2-1* cells were fully viable [data not shown]).

Since *dna2-2* cells are sensitive to MMS and bleomycin (14, 17, 19), we next tested the ability of *pif1Δ* to suppress the repair defect. The *pif1Δ* efficiently suppressed the MMS sensitivity of *dna2-2* (Fig. 1B).

In sum, the phenotypes of the *dna2-1 pif1Δ* and *dna2-2 pif1Δ* show that deletion of *PIF1* can suppress DNA replication and repair defects due to lesions in either the nuclease or the helicase domain of *DNA2*.

Deletion of *PIF1* suppresses the lethality of a *dna2Δ* strain. The suppression of temperature-sensitive growth of *dna2-1* and of the DNA damage sensitivity of *dna2-2* by deletion of *PIF1* suggested that *pif1Δ* might suppress the inviability of a deletion of *DNA2*. *PIF1* was disrupted in a *dna2Δ* strain containing a *DNA2*-complementing plasmid, pSEY18GALDna2 (7). Potential *dna2Δ pif1Δ*/pDNA2 double mutants were screened by testing for inability to grow on glycerol, since *pif1Δ* strains form *petites* at a high frequency. Deletion of *PIF1* was then confirmed by PCR and by complementation as described in Materials and Methods. *dna2Δ PIF1*/pDNA2 and *dna2Δ pif1Δ*/pDNA2 strains were then streaked on 5-fluoroorotic acid (5-FOA) plates to evict the Dna2-complementing plasmid, which carries *URA3*. The *dna2Δ pif1Δ* strain grew on the 5-FOA plates, whereas *dna2Δ PIF1* transformants did not. Serial dilutions of *dna2Δ pif1Δ* cells growing at 30°C are shown in Fig. 1B. Analysis of the *dna2Δ* strain in numerous crosses and tetrad dissections, as well as passage of the *dna2Δ pif1Δ* strain through genetic crosses described in the following experiments have never revealed an independently segregating suppressor. Therefore, deletion of *PIF1* suppresses the inviability of a deletion of *DNA2*.

Since suppression of a deletion of *DNA2* was unexpected, we used an alternative approach to confirm our results. A *PIF1*/*pif1Δ* heterozygote was transformed with a *kanMX* PCR product designed to disrupt the *dna2* gene (the *kanMX* gene flanked by 55 bp N terminal to the ATG and 55 bp C terminal to the stop codon of the *DNA2* gene) (34). *DNA2*/*dna2Δ*::*kanMX* heterozygotes were detected by PCR. The double heterozygote, ++/*pif1Δ dna2Δ* (MB504), was sporulated, and 36 tetrads were dissected. Thirty of the 94 viable spores recovered were *pif1Δ dna2Δ*, and none were *dna2Δ*. This is the expected number of spores if *DNA2* is essential and if *pif1Δ* suppresses the lethality of *dna2Δ*.

To test the efficiency of suppression, the doubling time of the *dna2Δ pif1Δ* double mutant was compared to that of a wild-type yeast. The doubling time at 30°C was 2.5 h, compared to 2 h for *pif1Δ*, suggesting that suppression was not due to complete bypass of the need for Dna2 (not shown). We also analyzed the ability of the *dna2Δ pif1Δ* mutant to grow at 37°C. The double mutant failed to form colonies at 37°C (data not shown here, but see Fig. 4 below), and cells arrested with dumbbell morphology characteristic of an S phase or G_2/M checkpoint (not shown). Therefore, deletion of *PIF1* suppresses the essential function of *DNA2* at 30°C but not at 37°C. We conclude that there is not complete bypass of Dna2 function, but suppression is nevertheless very strong.

Disruption of the nuclear, but not the mitochondrial function of *PIF1* suppresses *dna2Δ*. We wished to know whether it was disruption of the nuclear function and/or of the mitochondrial function of *PIF1* that allowed cells to grow in the absence of *DNA2*. Two alleles of *pif1*, *pif1-m1*, and *pif1-m2*, separate the nuclear and mitochondrial function of *PIF1* (50). *pif1-m1* has a mutation in the first ATG, so the protein is missing the mitochondrial import function and the strain is *petite*. *pif1-m1* appears to have its nuclear function intact, however, as evidenced by wild-type-length telomeres. *pif1-m2* has a mutation in the second ATG. The *pif1-m2* strain has its mitochondrial function intact but is deficient in its nuclear functions, leading to long telomeres. *pif1-m2* mutants also show increases in the rate of gross chromosomal rearrangements (GCRs) involving de novo telomere addition (36). *DNA2* was deleted from a set of isogenic strains containing mutations in *pif1*: 3615 (WT), 4400 (*pif1Δ*), 4393 (*pif1-m1*), 4344 (*pif1-m2*), and 5030 (*pif1-m2*) (36) by transforming each strain with a PCR product containing the *HIS3* gene and 55 bp N terminal to the ATG and 55 bp C terminal to the stop codon of the *DNA2* gene. The PCR product is expected to delete the *DNA2* gene in strains that do not require Dna2 for viability. No viable *dna2Δ pif1-m1* or *dna2Δ PIF1* transformants were recovered. Thus, disruption of the mitochondrial function of Pif1 does not suppress *dna2Δ* lethality. Conversely, *dna2Δ pif1Δ* and *dna2Δ pif1-m2* transformants, lacking the nuclear function of Pif1, were obtained and grew well, suggesting that it is the nuclear function of *PIF1* that creates a requirement for *DNA2* (Fig. 2A).

In order to evaluate the fitness of the *dna2Δ pif1-m2* mutant, its survival was tested at 37°C. As shown, the mutant was temperature sensitive, similar to the *dna2Δ pif1Δ* strain (Fig. 2B). The mutant was also replica plated to media containing 0.005% MMS, and the strain is also sensitive to MMS at 30°C (not shown). Thus, there is not total bypass of the requirement for Dna2 in the *pif1-m2* mutant in DNA replication or in repair.

Both the *dna2Δ pif1-m2* and the *dna2Δ pif1Δ* mutants grew more slowly than the *pif1-m2* mutant at 30°C. In studying the slow growth, 50 to 100 of the *dna2Δ pif-m2* transformants were replica plated to glycerol medium to determine if Dna2 deficiency led to production of *petites*. In fact, although the *pif1-m2* mutant grows normally on glycerol, the *dna2Δ pif1-m2* double mutants failed to grow on glycerol (Fig. 2C). This result may uncover a previously undiscovered role for Dna2 in mitochondrial maintenance, although further work is required to demonstrate that this is a direct effect. When a *dna2Δ pif1-m2 petite* mutant was crossed with *DNA2 PIF1 grande*, the diploids were all *grande*, suggesting that the *dna2Δ pif1-m2* mutant carried either defective (but not suppressive) mitochondrial genomes or lacked mitochondrial DNA entirely.

Failure of Rad53 checkpoint kinase activation in the *dna2-1 pif1Δ* double mutant. To investigate the extent to which *pif1Δ* removed the requirement for Dna2 in the cell, we indirectly measured in vivo DNA damage in *dna2* mutants in the presence and absence of Pif1 by determining the level of Rad53 phosphorylation. Rad53 is a protein kinase that is phosphorylated and activated in response to DNA damage and replication stress. In both *dna2-2* and in *dna2-1* mutants at permissive

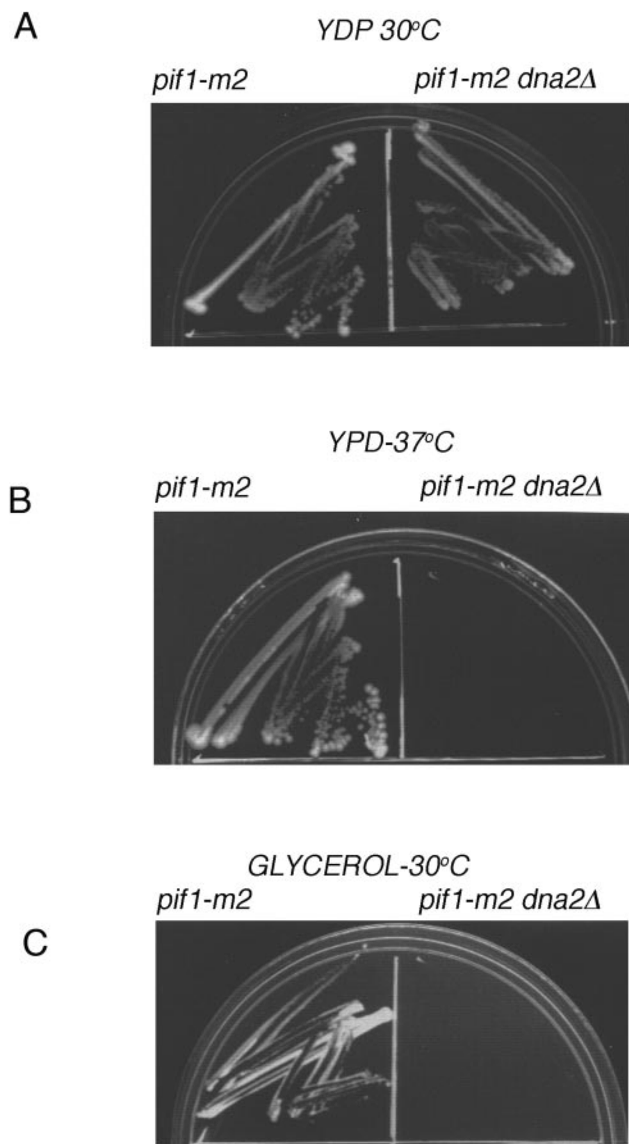

FIG. 2. Suppression of *dna2*Δ lethality by inactivation of Pif1 nuclear function but not by inactivation of Pif1 mitochondrial function. The following strains were used: 4344, *pif1-m2*; and 4344dna2Δ, *pif1-m2 dna2*Δ. (A and B) Growth on glucose-containing medium. (C) Growth on nonfermentable glycerol medium.

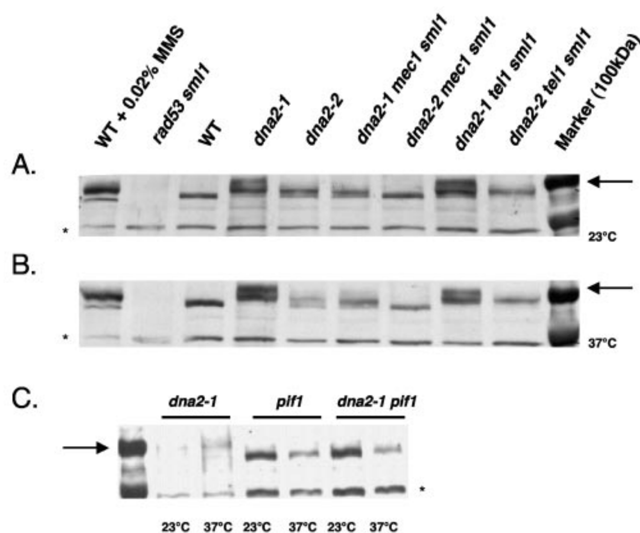

FIG. 3. Reduced DNA damage in *dna2-1 pif1*Δ. The strains used are isogenic derivatives of strain W303—MB90-7A, MB91, U953-61, MB92-M, SPY40, MB92-31C, MB92-35A, and U960-5C (Table 1)—with one exception, the *pif1*Δ strain MB509. The relevant genotypes are indicated in the figure. Strains were grown to approximately 1×10^7 per ml. Extracts were prepared and Western blots performed using antibody against Rad53 (gift of John Diffley, Clare Hall, England) as described previously (16). Wild-type W303 was also treated with 0.02% MMS where indicated to show phosphorylated Rad53. (A) Rad53 is phosphorylated in *dna2-1* and *dna2-2* strains at 23°C in a *MEC1*-dependent and *TEL1*-independent fashion. The 100-kDa marker runs with fully phosphorylated Rad53 and is indicated by the arrow in the figure. The asterisk denotes a nonspecific, cross-reacting species. (B) Rad53 is phosphorylated in *dna2-1* and *dna2-2* strains at 37°C in a *MEC1*-dependent and *TEL1*-independent manner. (C) *pif1*Δ suppresses Rad53 phosphorylation in the *dna2-1* strain.

and nonpermissive temperatures, Rad53 is indeed phosphorylated (Fig. 3A and B), consistent with *dna2* mutants suffering endogenous DNA damage. The phosphorylation, and implied DNA damage, seen at 23°C in the *dna2-1* mutant was not surprising since *dna2-1* mutants are sick even at this temperature. We have previously shown that the upstream master checkpoint kinase Mec1 is not essential for viability of *dna2-1* cells at the permissive temperature, and that *dna2-2 mec1*Δ *sml1-1* mutants grow more rapidly than *dna2-2* mutants (11). (Note that the *sml1-1* strain is included in the strains because it is required for *mec1*Δ viability.) We were therefore interested in whether *MEC1* was required for Rad53 phosphorylation in the *dna2-1* and *dna2-2* mutants. As shown in Fig. 3A and B, full Rad53 phosphorylation in the mutants is dependent on checkpoint induction by Mec1, since Rad53-P is drastically reduced in a *dna2-1 mec1*Δ *sml1-1* strain and absent in a *dna2-2 mec1*Δ *sml1-1* strain.

We have also previously shown that *dna2-2* mutants grow poorly in the absence of *TEL1*, a kinase related to *MEC1* (11). It is therefore interesting that Rad53 phosphorylation in *dna2-1* and *dna2-2* mutants is relatively independent of *TEL1*, compared to dependence on *MEC1* (Fig. 3A and B).

To see if there is less damage in a *dna2-1 pif1*Δ mutant, we measured Rad53 phosphorylation in the double mutant. As shown in Fig. 3C, the *dna2-1* mutant allele did not lead to induction of Rad53 phosphorylation, at either 23°C or 37°C, when *PIF1* was deleted. (The weaker, but clear signals in the *dna2-1* controls are due to loading less protein than in adjacent lanes, as indicated by the nonspecific protein indicated by the asterisk. Equivalent controls are also shown in Fig. 3A and B.) Since deletion of *PIF1* suppresses Rad53 phosphorylation in *dna2-1* mutants, we tentatively propose that there is less replication fork failure in the *dna2-1 pif1*Δ mutant than in the *dna2-1* mutant or that the checkpoint signal is somehow masked in the double mutant.

Suppression of the residual temperature sensitivity and MMS sensitivity in the *dna2*Δ *pif1*Δ mutant by *pol32*Δ. If Pif1 helicase creates 5′ flaps via its 5′-to-3′ helicase activity, then deletion of Pif1 should lead to reduced occurrence of 5′ flaps in the cell, which might, in part, account for the reduced re-

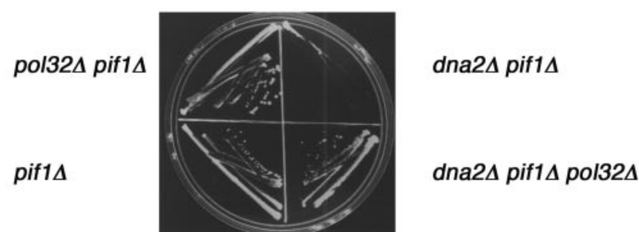

FIG. 4. Suppression of the temperature sensitivity of the *dna2Δ pif1Δ* strain by *pol32Δ*. The following strains were incubated on a YPD plate at 37°C for 3 days: MB202, *pif1Δ*; MB203.6, *dna2Δ pif1Δ*; MB206, *pif1Δ pol32Δ*; and MB207, *dna2Δ pif1Δ pol32Δ*.

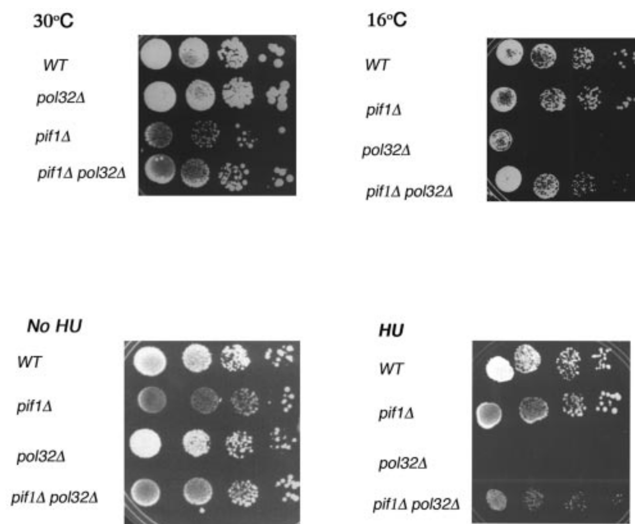

FIG. 5. Suppression of the cold sensitivity and HU sensitivity of the *pol32Δ* strain by *pif1Δ*. The following BY4741 isogenic strains were used: BY4741, WT; MB205, *pol32Δ*; MB201, *pif1Δ*; and MB206, *pol32Δ pif1Δ*. YPD plates were spotted with these strains and incubated at 30°C for 2 days (top, left panel) or at 16°C for 7 days (top right panel) as indicated. YPD plates were spotted with the same strains in the absence (lower left) or presence (lower right) of 38 mM HU and incubated at 23°C.

quirement for the 5′ flap helicase/nuclease activity of Dna2 in a *pif1Δ* mutant. *POL32* is a subunit of pol δ that leads to decreased 5′ flap strand displacement by pol δ when mutated (25). Since deletion of *POL32* also suppresses *dna2-1* temperature sensitivity (11), we asked whether deleting *POL32* could suppress the residual temperature-sensitive growth of the *dna2Δ pif1Δ* double mutant. Suppression was efficient, since the triple mutant now grew at 37°C (Fig. 4).

Genetic interaction between *PIF1* and *POL32*. In the course of these studies, we also obtained a *pifΔ pol32Δ* strain. Although the slow growth of the *pif1Δ* strain has always been attributed exclusively to the generation of *petites*, we noticed that the slow growth of the *pif1Δ* strain was suppressed by *pol32Δ* (Fig. 4; note colony sizes, for instance). While the *pif1Δ* strain grows more slowly than the *pol32Δ* strain, *pif1Δ pol32Δ* double mutants appear to have an intermediate growth rate between the *pol32Δ* strain, which grows like the wild type, and *pif1Δ* strains (Fig. 5, top, left). The putative suppression of *pif1Δ* strain slow growth by *pol32Δ* suggested that the *pif1Δ* mutation affects a nuclear function involving pol δ in addition to its mitochondrial defect. To further investigate the possible functional interaction between Pif1 and pol δ and to determine if it was related to the replication function of *POL32*, we tested if deletion of *PIF1* affects the cold-sensitive lethality and/or the hydroxyurea (HU; an inhibitor of DNA replication) sensitivity of *pol32Δ* strains. As shown in Fig. 5, the *pol32Δ* strain grows normally at 30°C. At 16°C, the *pol32Δ* strain is inviable, but viability is restored by deletion of *PIF1* (Fig. 5, top right). In addition, the *pol32Δ* strain is sensitive to inhibition of DNA replication by 38 mM HU, while the *pif1Δ pol32Δ* strain is resistant (Fig. 5, bottom). The suppression of the cold sensitivity and HU sensitivity of the *pol32Δ* strain by deletion of *PIF1* provides further evidence that Pif1 interacts with pol δ, most likely during chromosomal DNA replication.

Does Pif1 act through Dna2 to inhibit telomerase? While *pif1Δ* suppresses the requirement for Dna2, we also addressed the question of whether deletion of *DNA2* suppressed the requirement for Pif1. *pif1* mutants have long telomeres, overproduction of Pif1 results in short telomeres, and in vitro Pif1 reduces the processivity of telomerase, suggesting Pif1 acts as an inhibitor of telomerase (5, 50). Dna2 affects telomere metabolism in several ways. Dna2 is localized to telomeres in the G_1 and G_2 phases of the cell cycle but relocalizes during S phase to internal sites in the chromosomes, including but not limited to the rRNA gene (14). In addition, Dna2 is mobilized from telomeres upon treatment of cells with genotoxic agents such as bleomycin (14). Suggesting that telomeric localization implies a telomeric function for Dna2, we have shown that *dna2-2 est1Δ* mutants, lacking telomerase activity, senesce much more rapidly than *est1Δ* mutants, and *dna2-2 est1Δ* mutants show altered pathways of telomerase-independent survival (14). *dna2* mutants have normal or slightly long telomeres, but overproduction of *DNA2* leads to increased occurrence of single-stranded regions at telomeres (17, 39). Figure 6 compares telomere length in wild-type, *pif1Δ*, and *dna2Δ pif1Δ* strains. The *pif1Δ* telomere is longer than the wild-type telomere, as previously demonstrated (50). Interestingly, the *pif1Δ dna2Δ* telomere is dramatically shorter than the *pif1Δ* telomere and similar in size to the wild-type telomere. This suggests that the long telomere phenotype of *pif1Δ* is dependent on Dna2.

Given that *dna2-2 est1Δ* and *dna2-2 est2Δ* strains show increased rates of senescence compared to either single mutant, and given the reduction in telomere length in the *pif1Δ dna2Δ* mutant, we hypothesized that Dna2 might somehow aid telomerase in its function. If so, then we would expect *dna2Δ* to reduce the frequency of GCRs in a *pif1Δ* strain. We therefore investigated the effect of deletion of *DNA2* on the increased frequency of GCRs observed in *pif1-m2* mutants. The assay measures the rate of deletion of a region of chromosome V that is nonessential and that contains *CAN1* and *URA3*, by scoring the simultaneous appearance of canavanine-resistant (Can^R) 5-FOA^R cells. The *pif1-m2* mutant shows a 137-fold increase over wild-type strains in appearance of GCRs characterized primarily by the addition of new telomeres to internal chromosomal sequences (Table 2) (36). The rate of GCR formation was reduced to a 70-fold increase over the wild type in the *dna2Δ pif1-m2* strain (Table 2). This result is also consis-

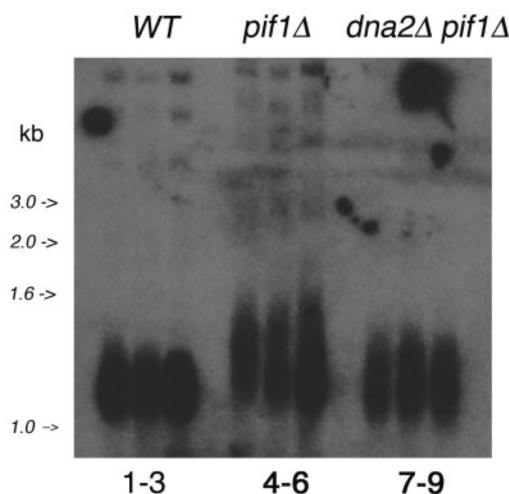

FIG. 6. Deletion of *DNA2* reduces telomere length in *pif1*Δ strains. Lanes 1, 2, and 3, WT strains 47421, BY4741, and BY4742, respectively. Lanes 4, 5, and 6, three colonies of *pif1*Δ strain 10509. Lanes 7, 8, and 9, *dna2*Δ *pif1*Δ strains MB203.3, MB203.5, and MB203.6, respectively. MB203.3, MB203.5, and MB203.6 are three independent *pif1*Δ transformants of MB110.

tent with an interaction between Pif1 and Dna2 in telomere biogenesis.

Mutations in genes that function to suppress GCRs usually lead to a greater than 100-fold increase in GCR frequency compared to the wild type (13, 36). Interestingly, *dna2-1* (a nuclease mutation), *dna2-2*, or *dna2 K1080A* (two different helicase mutations) caused only a 2- to 20-fold increase in GCRs compared to the wild type.

Interaction of *DNA2* and *PIF1* with other DNA2-interacting genes. We have recently completed a synthetic lethality screen with *dna2-1* and *dna2-2* and found that *DNA2* interacts with 56 genes affecting at least seven different genome maintenance pathways (11). We next tested whether the *pif1*Δ suppressed the synthetic effects previously observed between *dna2-1* and 27 of the 44 nonessential genes identified in the screen (Table 3). The genes chosen are representatives of each of the pathways identified. Deletion of *PIF1* failed to suppress the lethality of *dna2* with many genes involved in OFP, with other helicases, and with genes governing chromatin dynamics. The lethality of

TABLE 2. Frequency of GCRs

Relevant genotype[a]	GCR rate (Can^R 5-FOA^R)[b]
Wild type	3.5×10^{-10} (1)
pif1-m2	4.8×10^{-8} (137)
*pif1-m2 dna2*Δ	2.5×10^{-8} (71)
dna2-1	$>3.5 \times 10^{-10}$ (1)
dna2-2	7.0×10^{-9} (20)
dna2-K1080A	1.8×10^{-9} (5)

[a] All strains were constructed for this study by standard genetic techniques and are isogenic with the wild-type strain 3615 *MAT***a** *ura3-52 leu2*Δ*1 trp1*Δ*63 his3* Δ*200 lys2* Bgl *hom3-10 ade2-*Δ*1 ade8 hxt13*::*URA3*:*pif1-m2*, strain 4344; *pif1-m2 dna2*Δ, strain 4344dna2Δ; *dna2-1*, strain 3615-2-1; *dna2-2*, strain 3615-2-2; and *dna2-K1080A*, strain 3615-K1080A. The *dna2-K1080A* mutant is defective in helicase motif I.

[b] GCR rates were determined by fluctuation analysis of at least two experiments consisting of 5 or 11 cultures, and average values are reported. Numbers in parentheses indicate fold increase in GCRs compared to wild type.

TABLE 3. Growth of various triple mutants in the absence of Dna2 and Pif1[a]

Effect	*dna2*Δ *pif1*Δ genes
Lethal	*hst3, rtf1, sae2, mre11, yen1, exo1, rnh35, rad27, rrm3, ctf4, sgs1, bre1*
Sick	*est2,*[b] *rpd3, rtt103, sap30, lge1, swd3, swd1, vid21*
No effect	*cdc73, caf20, hir3, htz1, paf1, rad18, rad51*

[a] All mutations are synthetic lethal with *dna2-1* and/or *dna2-2.* All mutations are deletions in the BY4741 background and were obtained by crossing MB203.3 with strains carrying the respective single mutations obtained from Invitrogen. Tetrad analysis was performed by standard techniques. At least 10 tetrads were dissected for each cross. Synthetic lethality was scored as the absence of viable triple mutant spores. Synthetic sickness was also scored and represents fewer than the expected number of triple mutants and slower growth than either the single mutant or the *dna1*Δ *pif1*Δ strain.

[b] Enhanced senescence.

the triple mutants rules out a potentially trivial explanation of the synthetic lethality of these genes with *dna2-1*, namely, that transcription of *dna2-1* was reduced by the mutations. The lethality of the triple mutants also is consistent with other results presented here that indicate that *pif1*Δ does not fully bypass the need for Dna2.

Usefulness of *dna2*Δ *pif1*Δ: synthetic lethality of *rtf1*Δ but not of *paf1*Δ with *dna2*Δ *pif1*Δ. Recently, we reported that *dna2-1* was synthetically lethal with members of the Rad6 epistasis group specifically involved in histone modification. *dna2-1* was synthetically lethal with *rtf1*, a gene encoding a member of the PAF1 transcription factor/histone modification complex (11). *dna2*Δ *pif1*Δ *rtf1*Δ mutations are also synthetically lethal (Table 3). To try to distinguish whether the effect was indirect and due to reduced transcription of another replication gene, we tested whether *dna2*Δ *pif1*Δ was synthetic lethal with another member of the pathway, *paf1*Δ. We had previously found that *dna2-1 paf1*Δ mutant was viable, but were concerned that the viability might result from residual Dna2 protein activity in the *dna2-1* strain. Indeed, *dna2*Δ *pif1*Δ *paf1*Δ triple mutants were viable. The difference between the effects of deletion *PAF1* and *RTF1* shows that synthetic sickness with one member of a pathway does not imply synthetic sickness with additional members, likely due to the participation of individual components in multiple, nonoverlapping pathways. The difference between the effect of *paf1* and *rtf1* correlates, not with an effect on transcription, but with their quantitative effect on methylation. *paf1* mutants reduce dimethylation of histone H3 by 75%, while dimethylation is nearly completely eliminated in the *rtf1* mutant. Therefore, the synthetic lethality of *rtf1* with *dna2* is more likely due to a histone methylation defect than to a transcription defect. This interpretation is also supported by recent evidence that Rtf1 has an additional role in methylation in a process other than transcription elongation (38). Because *dna2*Δ *pif1*Δ is synthetically lethal with several other histone modification gene deletions (Table 2) (11), we propose that the synthetic lethality of *dna2*Δ *pif1*Δ *rtf1*Δ is due to failure of dimethylation of histone H3 K4.

DISCUSSION

We have shown that deletion of *PIF1* or inactivation of its nuclear function suppresses the temperature-sensitive pheno-

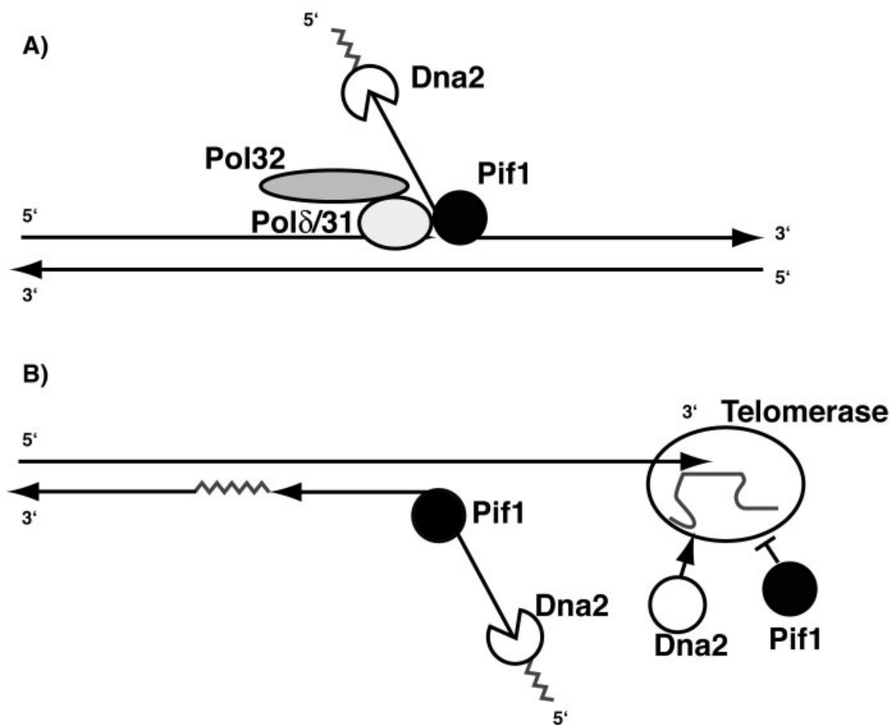

FIG. 7. Interpretation of genetic interactions between *PIF1* and *DNA2*. (A) Pif1 may help to make a flap during RNA removal by Dna2 during OFP. (B) Dna2 may be required for optimum elongation of telomeres by telomerase, whereas Pif1 has been shown to inhibit telomerase processivity (5). Pif1 may have a second role at telomeres in which it aids Dna2 in removal of RNA from the Okazaki fragments at the telomere. The last Okazaki fragment might have a special requirement for Pif1 helicase as there is no pol δ/PCNA present to strand displace and to recruit FEN1.

type of *dna2-1* mutants, the MMS sensitivity of *dna2-2* mutants, and even the inviability of *dna2*Δ mutants. Our main conclusions are summarized in Fig. 7, namely that Pif1 is involved in OFP and that Dna2 is involved, along with Pif1, in regulating telomere length. We will discuss three primary insights underlying these conclusions. First, the suppression of the lethality of the *dna2*Δ mutation by *pif1*Δ shows that the nuclear function of Pif1 creates the essential requirement for Dna2, a helicase/nuclease involved in Okazaki fragment processing. In molecular terms, this first suggests that the Pif1 helicase generates structures that are lethal to the cell if Dna2 is not present. Similarly, the absence of Dna2 may lead to structures that are lethal if processed by Pif1. Second, the genetic interaction between *PIF1* and *DNA2* implies that the nuclear form of Pif1 may play a more general role in chromosomal replication than previously thought. The related observation that *pif1*Δ also suppresses the cold sensitivity and the HU sensitivity of *pol32*Δ, a subunit of pol δ, a major DNA polymerase at the replication fork, supports this interpretation. Third, the suppression of the major *pif1*Δ phenotype, long telomeres, by *dna2*Δ shows that the long telomeres in *pif*Δ mutants require Dna2 for synthesis.

***PIF1* creates a requirement for *DNA2*.** Dna2 is thought to participate in primer removal from Okazaki fragments, but only in cases where pol δ strand displaces more than 30 bases of the downstream Okazaki fragment before ligation (2, 24, 28, 29). Dna2 is a 5′-to-3′ helicase and a nuclease that requires a single-stranded DNA end for activity (29). Dna2 appears to load onto a single-stranded terminus and to translocate by a threading mechanism (27). Thus, Dna2 has evolved to be compatible with processing displaced flaps but not to cleave single-stranded regions endonucleolytically. In addition to the biochemical properties of Dna2, genetic interactions with other genes that function on 5′ flaps, such as those coding for FEN1, Exo1, and Yen1, and with genes involved in removing RNA, such as that coding for RNase H2, strengthen the notion that Dna2 is required for OFP (8, 11). Thus, although Okazaki fragments have not formally been demonstrated to accumulate in *dna2* mutants, there is overwhelming biochemical and genetic evidence that the essential function of Dna2 lies in some aspect of OFP. Taking that as our assumption, our new results suggest that one substrate of Pif1 is an Okazaki fragment. There are several ways in which Pif1 might function: either through a mechanism intrinsic to Okazaki fragment formation and maturation or, more indirectly, in repairing faulty synthesis or processing. In OFP, reconstitution of reaction mixtures containing pol δ, replication protein A (RPA), Dna2, FEN1, DNA ligase, and model substrates representing intermediates in OFP, suggests that Dna2 is required for OFP only when there is excessive (greater than 30 nucleotides) strand displacement of a 5′ flap on the previously synthesized Okazaki fragment by the pol δ extending the nascent fragment. The role of Dna2 helicase/nuclease is to participate in removal of the flap. This situation probably does not arise at every Okazaki fragment, however, since pol δ and FEN1 normally act in concerted fashion producing only transient flaps shorter than 4

nucleotides in vitro (18). Furthermore, Dna2 is not essential in vitro even to remove long flaps in the presence of FEN1, but is only stimulatory to FEN1, which appears to be the major nuclease. Our studies suggest that in this strand displacement pathway for OFP, there may be an additional participant, Pif1 helicase. Pif1 might assist pol δ in strand displacement, and a *pif1* mutation may reduce requirement for Dna2 by reducing levels of strand displacement.

We have previously shown that *dna2* mutants grow better in the absence of *MEC1* but have increased growth defects in the absence of *TEL1* (11). This implies that there is sufficient damage to induce the Mec1-dependent checkpoint in *dna2* cells, but that the checkpoint is not necessary for viability. Instead, the checkpoint slows growth, perhaps to allow repair. We verify that result here with physical evidence that Rad53 becomes phosphorylated in *dna2-1* and *dna2-2* mutants and that Rad53 phosphorylation is dependent on Mec1 kinase but not on Tel1 kinase. However, in the *dna2-1 mec1Δ sml1-1* strain (Fig. 3A), there is still some phosphorylation, unlike in the *dna2-2 mec1Δ sml1-1* strain. The two different mutations could generate different types of DNA damage. In any case, deletion of *PIF1* resulted in drastic reduction of the Rad53 phosphorylation, which we propose would be due to a reduction in extended flaps below the threshold required to activate the checkpoint, also suggesting a tight connection between Pif1 and Dna2 function in DNA replication.

It remains possible that Pif1 is only required for repair, although we think it less likely. In this scenario, Pif1 would create a DNA structure at sites where DNA replication forks on the lagging strand are blocked by endogenous DNA damage, and this structure is lethal if not resolved. Dna2 helicase/nuclease would then be required for removal of this structure.

Genetic interactions between *PIF1*, *DNA2*, and *POL32* suggest a general (but not universal) role for *PIF1* in replication fork progression. Several of our recent results support the model just presented in which Dna2 acts on flaps produced by both pol δ and Pif1. First, the temperature sensitivity of *dna2-1* mutants and the DNA damage sensitivity of *dna2-2* mutants, but not the lethality of *dna2Δ*, are suppressed by the deletion of *POL32* (11). The *POL32* gene encodes a subunit of pol δ that is required for optimum processivity, and its deletion can be expected to reduce strand displacement in vivo as it has been demonstrated to do in vitro (12, 25). This would explain the reduced requirement for *DNA2*. Second, the *dna2-1* mutation is synthetically lethal with *pol3-01* (11), a pol δ mutation expected to increase strand displacement (18). This would explain the synthetic lethality. Third, the observation presented in this work of the suppression of residual defects in the *dna2Δ pif1Δ* strain by deletion of *POL32* is consistent with a model in which deletion of Pif1 reduces but does not abolish strand displacement, while further deletion of *POL32* eliminates strand displacement and shifts the course of Okazaki fragment processing to a pathway that is Dna2 independent, such as an RNase H/FEN1-alone pathway (33). As would be anticipated in such a model, *dna2-1* and *dna2Δ pif1Δ* are synthetically lethal with genes encoding RNase H2 subunits (*rnh35Δ* and *rnh202Δ*) (11) (Table 2). This putative Dna2-independent pathway might be inhibited by flap production by Pif1, since we have shown here that deletion of *PIF1* also suppresses the cold-sensitive lethality of *pol32Δ*, which in itself might favor the alternative Dna2-independent pathway. Finally, suppression of the slow growth of *pif1Δ* by deletion of a purely nuclear function, *POL32*, suggests that *PIF1* is required for optimal nuclear replication and not just for mitochondrial DNA stability, as previously proposed. In a more general sense, the nature of the genetic interactions between *PIF1*, *POL32*, and *DNA2* suggest that a delicate balance of these three activities is required for maintaining chromosomal stability in a range consistent with viability. Since such a role in nuclear replication for *PIF1* has been overlooked to date, further biochemical reconstitution experiments incorporating Pif1 helicase and pol δ lacking Pol32 will be required to fully understand this pathway.

Although it is widely held that Pif1 functions in chromosomal DNA replication primarily at telomeres, the genetic interactions we report, if indeed they do imply nontelomeric activities, are not the first evidence that *PIF1* does not function solely at telomeres in *S. cerevisiae*. Ivessa et al. showed clearly that Pif1 is required for replication fork pausing at the Fob1-dependent replication fork barrier in the rRNA gene (22). Dna2, in contrast, is required to prevent pausing, and in its absence, there is not only increased pausing but also an increased frequency of DSBs at the RFB (47, 48). Thus, Dna2 and Pif1 may have opposing functions at the RFB, just as they appear to have at telomeres (Fig. 6).

***DNA2* in chromosomal, mitochondrial, and telomere replication.** Although deletion of *PIF1* suppresses the lethality of a *dna2Δ* mutation and the DNA damage/replication stress-induced Rad53 phosphorylation seen in *dna2* mutants, mutants retain defects in DNA replication and repair since they are temperature sensitive and sensitive to MMS. We have begun to explore the residual DNA damage in the *dna2Δ pif1Δ* double mutant. Analysis of cell cycle progression and DNA replication in synchronized cells after ectopic expression of *PIF1* in a *dna2Δ pif1Δ* background revealed that cells arrested within the first cell cycle as dumbbells, a G_2/M arrest (data not shown). Further work should allow a more sensitive evaluation of the precise steps in DNA replication that require Dna2 than were possible with conditional alleles, which are by definition leaky.

The *DNA2* gene can be deleted in the *pif1-m2* strain, which has a mutated nuclear and functional mitochondrial Pif1, but cannot be deleted in a *pif1-m1* strain, which has a functional nuclear and mutated mitochondrial Pif1. We noticed, however, that all of the *dna2Δ pif1-m2* strains are *petite*. Since *pif1-m2* is not deficient in mitochondrial DNA functions, Dna2 might be required for mitochondrial DNA stability. The mechanism of yeast mitochondrial DNA replication is not well enough understood, however, to speculate on whether the effect is due to a direct role for Dna2 in mitochondrial DNA metabolism or if it is indirect.

Pif1 has been proposed to inhibit telomerase, since *pif1Δ* mutants have long telomeres and show increased frequency of de novo telomere synthesis at internal DSBs (50). DSBs give rise to a high frequency of GCRs in *pif1-m2* strains (36). Although Pif1 inhibits telomerase in vitro (5), the mechanism of telomerase inhibition in vivo is not fully understood. The fact that we show that deletion of *DNA2* in a *pif1Δ* strain restores nearly normal telomere length suggests that Dna2 may need to be functional in order to observe long telomeres in the absence of Pif1. Dna2 may function at telomeres to stimulate telomerase-dependent telomere lengthening (Fig. 4 and 7).

This interpretation is consistent with our previous work (see the introduction and reference 14), but the putative activation role of Dna2 on telomerase synthesis is more directly unmasked by deletion of *PIF1*, as shown here. The reduction in GCR rate from a 137-fold increase over wild type in the *pif1-m2* strain to a 70-fold increase in the *pif1-m2 dna2*Δ double mutants also is consistent with this interpretation.

The *dna2*Δ *pif1*Δ telomere phenotype is similar to that of *rrm3*Δ *pif1*Δ. The long telomeres of the *pif1*Δ mutant and the frequency of telomere addition to DSBs in *pif1*Δ cells require *RRM3* (20). Also, the GCR rate of *pif1*Δ is reduced by deletion of *RRM3* (20). The *dna2*Δ *rrm3*Δ *pif1*Δ triple mutant is inviable (Table 2), obviating an experiment to test if telomeres are even shorter or the GCR rate is further reduced than in either double mutant. Outside of *rrm3*Δ and mutations that reduce telomerase activity (e.g., *est2*Δ and *stn1-13*), very few mutations have been found that reduce the GCR rate in *pif1-m2* strains (36, 37). These comprise spindle checkpoint genes (*mad2*, *mad3*, and *bub3*), a mitotic exit network gene (*bub2*), and sister chromatid cohesion genes (*ctf8*, *ctf18*, and *dcc1*) (37). These genes may be involved in allowing cells with pre-GCR lesions to survive and generate GCRs. *dna2*Δ is not known to affect the spindle checkpoint. Thus, Dna2 suppression of GCRs in the *pif1-m2* strain is more similar to suppression by *rrm3*Δ, *stn1-13*, and *est2*Δ. A further connection between Dna2 and telomerase-dependent telomere elongation is provided by the fact that *dna2*Δ *pif1*Δ *est2*Δ cells form small colonies and senesce more rapidly than *est2*Δ mutants (Table 2).

Diede and Gottschling (15) have shown that telomerase does not synthesize telomeres de novo in the absence of Okazaki fragment synthesis. The effects of Dna2 and/or Pif1 on telomere synthesis may be a consequence of a role in OFP (Fig. 7). If Pif1 inhibits telomerase indirectly as well as directly, it might do so by functioning on the lagging strand during telomere maturation. Pif1 may displace the strand on the Okazaki fragment at the end of the telomere, exposing G-rich DNA, which may potentially form a G-quartet structure that inhibits telomerase. These G-quartets might not hybridize to the telomerase RNA, for instance. The absence of Pif1 may minimize formation of such structures. If Pif1 functions as an inhibitor of telomerase independently of Dna2, then a telomerase lacking Pif1 inhibition still requires Dna2 for optimal function, since telomeres are shortened in the *dna2*Δ *pif1*Δ mutant. If a major role of Dna2 is at telomeres, this might explain some puzzling observations in *C. elegans*. All fungal versions of *DNA2* are essential. *C. elegans dna2* mutants show a more complex phenotype. They appear deficient in DNA replication but show 90% embryonic viability in F_1. However, they are embryonic lethal in F_2 (32). Late-generation lethality is a phenotype of telomere deficiency in other organisms.

In *S. pombe*, *pfh1*$^+$ is an essential helicase whose helicase domain is closely related to both the nonessential *S. cerevisiae* Pif1 and Rrm3 proteins, but which diverges from both in the N terminus. It has been difficult to make a one-to-one comparison with respect to function between *pfh1*$^+$ and either *RRM3* or *PIF1* to date. Our demonstration that *S. cerevisiae PIF1* shows genetic interaction with *DNA2* and *POL32* may shed light on the conserved functions of *PIF1*, since *S. pombe pfh1*$^+$, *dna2*$^+$, and *cdc27*$^+$ (the ortholog of *POL32*) also interact genetically. A mutant allele of *pfh1*$^+$, *pfh1-R20*, affecting the ATPase domain, suppresses the temperature-sensitive growth of the *dna2-C2* mutant, which has a mutation affecting the Dna2 helicase domain of the *S. pombe dna2*$^+$ strain (41, 45). Whether the *pfh1* mutant suppresses nuclease-deficient *S. pombe dna2* strains, as *pif1*Δ suppresses the *dna2*Δ and *dna2-1* (a lesion in the nuclease) mutants of *S. cerevisiae*, has not been reported. Nor has it been shown that deletion of *pfh1* bypasses the essential function of *DNA2*, as no nuclease-deficient *dna2* alleles have been described in *S. pombe*. Therefore, a direct interaction between the two is less well established in *S. pombe*. *S. pombe pfh1*$^+$ and *cdc27*$^+$ also interact. In contrast to the suppression of *S. cerevisiae pol32*Δ by *pif1*Δ that we observe, however, in *S. pombe*, *cdc27* conditional mutants are synthetically lethal with *pfh1-R20* and *pfh1-R23* mutations (44). The *pfh1* mutations may affect a function performed in *S. cerevisiae* by *RRM3* rather than by *PIF1*, accounting for the difference between the two organisms. Another difference between the two organisms is that an additional essential gene, *cdc24*$^+$, which has not been identified in *S. cerevisiae*, appears to interact with these genes in *S. pombe*. Despite the differences in alleles tested to date in the two organisms, it is clear that the essential *pfh1*$^+$ gene in *S. pombe* shows similar genetic interactions to *PIF1*, and therefore it appears that it is *S. cerevisiae PIF1*, and not *RRM3*, as previously believed, that conserves the putative essential role of *pfh1*$^+$ in interactions with *DNA2* and with pol δ in OFP.

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