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Telomerase Is Essential to Alleviate Pif1-Induced Replication Stress at Telomeres

Michael Chang,*,†,‡,1 Brian Luke,†,‡ Claudine Kraft,§ Zhijian Li, Matthias Peter,§ Joachim Lingner†,‡ and Rodney Rothstein***

**Department of Genetics and Development, Columbia University Medical Center, New York, New York 10032, †Swiss Institute for Experimental Cancer Research, École Polytechnique Fédérale de Lausanne, CH-1015 Lausanne, Switzerland, ‡National Centre of Competence in Research "Frontiers in Genetics," CH-1211 Geneva, Switzerland, §Institute of Biochemistry, Swiss Federal Institute of Technology, CH-8093 Zürich, Switzerland and **Banting and Best Department of Medical Research and Department of Molecular Genetics, Terrence Donnelly Centre for Cellular and Biomolecular Research, University of Toronto, Toronto, Ontario M5S 3E1, Canada*

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ABSTRACT

Pif1, an evolutionarily conserved helicase, negatively regulates telomere length by removing telomerase from chromosome ends. Pif1 has also been implicated in DNA replication processes such as Okazaki fragment maturation and replication fork pausing. We find that overexpression of *Saccharomyces cervisiae PIF1* results in dose-dependent growth inhibition. Strong overexpression causes relocalization of the DNA damage response factors Rfa1 and Mre11 into nuclear foci and activation of the Rad53 DNA damage checkpoint kinase, indicating that the toxicity is caused by accumulation of DNA damage. We screened the complete set of ∼4800 haploid gene deletion mutants and found that moderate overexpression of *PIF1*, which is only mildly toxic on its own, causes growth defects in strains with mutations in genes involved in DNA replication and the DNA damage response. Interestingly, we find that telomerase-deficient strains are also sensitive to *PIF1* overexpression. Our data are consistent with a model whereby increased levels of Pif1 interfere with DNA replication, causing collapsed replication forks. At chromosome ends, collapsed forks result in truncated telomeres that must be rapidly elongated by telomerase to maintain viability.

Pif1 is a 5′–3′ helicase that is evolutionarily conserved from yeast to humans (Boule and Zakian 2006). It was first identified in the budding yeast *Saccharomyces cerevisiae* for its role in mitochondrial DNA maintenance as cells lacking Pif1 lose mitochondrial DNA at high rates, generating respiratory-deficient (petite) cells (Foury and Kolodynski 1983; Lahaye *et al.* 1991). Cells also express a nuclear form of Pif1 that has functions independent of mitochondrial DNA maintenance, with its role in telomerase regulation being the most thoroughly characterized.

Telomeres, the physical ends of eukaryotic chromosomes, protect chromosome ends from end fusions and degradation (Ferreira *et al.* 2004). Telomere length is maintained by a dynamic process of lengthening and shortening (Teixeira *et al.* 2004). Shortening occurs by a combined result of nucleolytic degradation and incomplete DNA replication. Lengthening is primarily accomplished by the action of the reverse transcriptase telomerase, whose catalytic core consists of a protein subunit and an RNA moiety (Est2 and TLC1, respectively, in *S. cerevisiae*) (Singer and Gottschling 1994; Lendvay *et al.* 1996; Lingner *et al.* 1997).

*pif1*Δ mutants have long telomeres, while overexpression of *PIF1* leads to modest shortening of telomeres (Schulz and Zakian 1994; Zhou *et al.* 2000). *De novo* telomere addition at double-stranded DNA breaks (DSBs) is increased 600- to 1000-fold in cells lacking Pif1 (Schulz and Zakian 1994; Mangahas *et al.* 2001; Myung *et al.* 2001). These phenotypes are dependent upon telomerase, suggesting that Pif1 directly inhibits telomerase both at naturally occurring telomeres and at DSBs. Indeed, Pif1 can remove telomerase from its DNA substrates both *in vivo* and *in vitro* (Boule *et al.* 2005). Furthermore, Pif1 preferentially unwinds RNA–DNA hybrids, consistent with a model where Pif1 displaces telomerase by unwinding the RNA–DNA hybrid formed between TLC1 and the telomeric DNA overhang (Boule and Zakian 2007).

Like its yeast counterpart, human Pif1 (hPif1) can be found both in the mitochondria and in the nucleus (Futami *et al.* 2007), and ectopic expression of hPif1 causes telomere shortening (Zhang *et al.* 2006). hPif1 interacts with telomerase, associates with telomerase activity (Mateyak and Zakian 2006), reduces telomerase processivity, and can unwind a DNA–RNA duplex *in vitro* (Zhang *et al.* 2006). Mouse Pif1 also interacts with telomerase, although it appears to be dispensable for telomere function *in vivo* (Snow *et al.* 2007).

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[1]*Corresponding author:* 701 W. 168th St., New York, NY 10032. E-mail: mc3096@columbia.edu

In *S. cerevisiae*, overexpression of *PIF1* reduces the viability of *yku70*, *yku80*, and *cdc13* mutants (Banerjee *et al.* 2006; Vega *et al.* 2007) while absence of Pif1 rescues the temperature sensitivity of these mutants (Downey *et al.* 2006; Vega *et al.* 2007; Smith *et al.* 2008). Yku70 and Yku80 form the heterodimeric Ku complex that protects telomeres from degradation and inappropriate recombination (Gravel *et al.* 1998; Polotnianka *et al.* 1998; Dubois *et al.* 2002; Maringele and Lydall 2002). Cdc13, an essential protein that binds to the G-rich telomeric single-stranded 3′ overhangs, is also required for chromosome end protection (Garvik *et al.* 1995; Nugent *et al.* 1996). Mutations in *YKU70*, *YKU80*, and *CDC13* can result in extensive resectioning of the C-rich strand, leaving long G-rich overhangs. Additionally, Ku and Cdc13 are important for recruiting telomerase to telomeres (Pennock *et al.* 2001; Stellwagen *et al.* 2003; Fisher *et al.* 2004) and Ku is also important for nonhomologous end joining (NHEJ) (Fisher and Zakian 2005). However, the sensitivity of *yku* and *cdc13* mutants to *PIF1* overexpression is directly related to the fact that these mutants have long G overhangs (Vega *et al.* 2007). Therefore it was proposed that the levels of telomere-bound telomerase are important for the viability of mutants with defective telomere capping (Vega *et al.* 2007).

Nuclear Pif1 also has additional roles outside of telomerase regulation. Pif1 helps to maintain the replication fork barrier in the ribosomal DNA (rDNA) repeats (Ivessa *et al.* 2000). Furthermore, deletion of *PIF1* suppresses the lethality associated with a deletion of *DNA2*, a gene involved in Okazaki fragment maturation (Budd *et al.* 2006). Likewise, *Schizosaccharomyces pombe pfh1+* (*PIF1* homolog) has similar genetic interactions with genes involved in Okazaki fragment processing (Tanaka *et al.* 2002; Zhou *et al.* 2002; Ryu *et al.* 2004). It has been suggested that Pif1, together with DNA polymerase δ, helps generate flaps from the 5′ ends of Okazaki fragments (Ryu *et al.* 2004; Budd *et al.* 2006; Boule and Zakian 2007; Rossi *et al.* 2008; Stith *et al.* 2008). Pif1 also counteracts Sgs1 DNA helicase activity, promoting survival in the absence of the Top3 topoisomerase (Wagner *et al.* 2006), and may also play a role in preventing genomic instability caused by G-quadruplex-forming sequences (Ribeyre *et al.* 2009).

In this study, we show that overexpression of *PIF1* inhibits growth in a dose-dependent fashion. The growth inhibition can be attributed to DNA damage, as evidenced by Rad53 checkpoint kinase activation, requirement of checkpoint activity for viability, and the relocalization of the DNA damage factors Rfa1 and Mre11 into nuclear foci. This damage is present at, but not specifically restricted to, telomeres. In addition, it is likely caused by interference with lagging-strand DNA replication and is independent of Pif1's role as a negative regulator of telomerase. Unexpectedly, telomerase activity is required for viability when *PIF1* is overexpressed, indicating that the damage present at telomeres is repaired by telomerase. We propose a model whereby overexpression of *PIF1* causes replication defects, which at telomeres results in replication fork collapse that requires repair by telomerase.

MATERIALS AND METHODS

Yeast media, strains, and plasmids: Standard yeast media and growth conditions were used (Sherman 1991). Yeast strains used in this study are listed in Table 1. Nonessential haploid deletion strains were made by the Saccharomyces Gene Deletion Project (Giaever *et al.* 2002). ρ^0 petite strains lacking mitochondrial DNA were isolated by growing cells in the presence of ethidium bromide.

The 2-μm plasmid containing a fusion ORF encoding protein A-, hemagglutination-, and 6× histidine-tagged Pif1 under the control of the *GAL1* promoter (BG1805-PIF1) was constructed as part of a 2-μm ORF collection (Gelperin *et al.* 2005) and purchased from Open Biosystems. The BG1766 vector control was a gift from Elizabeth Grayhack. The centromeric plasmids pVS45 (expressing the nuclear form of Pif1 under the control of the *GAL1* promoter) and pSH380 (a pRS315-derived vector control) were kindly provided by Virginia Zakian (Vega *et al.* 2007). The pSE358-EST2 and pSE358-est2D670A plasmids were gifts from Neal Lue. pRS313-est2-up34 was a gift from Eric Gilson (Eugster *et al.* 2006).

Flow cytometry: A total of 750 μl of logarithmically growing cells was harvested and fixed in 70% ethanol. Samples were washed once with water, resuspended in 0.2 mg/ml RNaseA in 50 mm Tris-Cl (pH 8.0), and incubated at 37° for 4 hr. Samples were then harvested, resuspended in 50 mm Tris-Cl (pH 7.5) containing 2 μg/ml proteinase K, and incubated at 50° for 1 hr. Samples were harvested again and resuspended in 0.5 ml of FACS buffer [200 mm Tris-Cl (pH 7.5), 200 mm NaCl, 78 mm $MgCl_2$]. A total of 50 μl was transferred into a tube containing 1 ml of 50 mm Tris-Cl (pH 7.5) containing Sytox Green (Molecular Probes, Eugene, OR). The samples were sonicated briefly and analyzed using a Becton Dickinson FACSCalibur.

Rad53 *in situ* kinase assays and immunoblotting: Rad53 *in situ* kinase assays were performed essentially as described (Pellicioli *et al.* 1999). To simultaneously detect Rad53 (data not shown) and overproduced protein A-tagged Pif1, proteins were separated on 7.5% polyacrylamide–SDS gels, and the immunoblots were probed with anti-RAD53 (yC-19, Santa Cruz).

Fluorescence microscopy: Cells expressing Rfa1-CFP and either Rap1-YFP or Mre11-YFP were grown to logarithmic phase at 23° in SC medium. Microscopy was performed essentially as described (Lisby *et al.* 2004).

Synthetic dosage lethality screen: The *S. cerevisiae* heterozygous diploid gene deletion mutant array (Open Biosystems) was replica pinned onto sporulation medium. Sporulated cells were mixed with strain BY7220 transformed with the plasmid pVS45. Synthetic genetic array (SGA) methodology was then used to isolate haploid gene deletion mutants containing pVS45 (Tong *et al.* 2004; Tong and Boone 2006). Cells were replica pinned onto media containing either glucose or galactose. Mutants that grew poorly on galactose were verified for sensitivity to moderate *PIF1* overexpression by reintroducing pVS45 or the vector control pSH380 into each strain by traditional yeast transformation methods, followed by spotting 10-fold serial dilutions of yeast culture onto media containing either glucose or galactose.

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TABLE 1

Yeast strains used in this study

Strain name	Genotype	Source
BY4741	*MAT***a** *his3Δ1 leu2Δ0 ura3Δ0 met15Δ0*	Brachmann *et al.* (1998)
MCY236	*MAT***a** *rad53-11::URA3 his3Δ1 leu2Δ0 ura3Δ0 met15Δ0*	Bellaoui *et al.* (2003)
W8550-5A	*MAT*α *RFA1-CFP RAP1-YFP ADE2 RAD5 his3-11,15 leu2-3,112 trp1-1 can1-100 ura3-1*	This study
W8705-1A	*MAT***a** *RFA1-CFP MRE11-YFP ADE2 RAD5 his3-11,15 leu2-3,112 TRP1 lys2Δ ura3-1*	This study
BY7220	*MAT*α *can1::STE2pr-Sp_his5 lyp1Δ cyh2 his3Δ1 leu2Δ0 ura3Δ::natMX met15Δ*	Charlie Boone
Y8856	*MAT***a** *pol1-1::kanMX his3Δ1 leu2Δ0 ura3Δ0 met15Δ0*	This study
Y8875	*MAT***a** *pol12-100::kanMX his3Δ1 leu2Δ0 ura3Δ0 met15Δ0*	This study
Y4855	*MAT***a** *pri2-1::kanMX his3Δ1 leu2Δ0 ura3Δ0 met15Δ0*	This study
Y7243	*MAT***a** *cdc2-2::kanMX his3Δ1 leu2Δ0 ura3Δ0 met15Δ0*	This study
Y8849	*MAT***a** *dna2-1::kanMX his3Δ1 leu2Δ0 ura3Δ0 met15Δ0*	This study
Y5129	*MAT***a** *cdc9-1::kanMX his3Δ1 leu2Δ0 ura3Δ0 met15Δ0*	This study
Y10155	*MAT***a** *stn1-13::kanMX his3Δ1 leu2Δ0 ura3Δ0 met15Δ0*	This study
YBJ1	*MAT*α *ura3-52 leu2-3,112 his3-200 ade2-101 lys2-801*	Brad Johnson
YBJ120	*MAT*α *cdc13-1 ura3-52 leu2-3, 112 his3-200 ade2-101 lys2-801*	Brad Johnson
U1374	*MAT***a**/α *EST2/est2Δ::URA3 ade2-1/ade2-1 can1-100/can1-100 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 trp1-1/trp1-1 ura3-1/ura3-1 RAD5/RAD5*	Marisa Wagner
YBL312	*MAT***a** *rad50ΔkanR his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0*	This study
YBL618	*est2ΔnatR rad50ΔkanR his3Δ1 leu2Δ0 ura3Δ0* (type I survivor)	This study
YBL311	*MAT***a** *est1ΔkanR his3Δ1 leu2Δ0 ura3Δ0* (type II survivor)	This study
YBL314	*est1ΔkanR his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0* (type II survivor)	This study
YBL613	*MAT***a** *est2ΔnatR his3Δ1 leu2Δ0 ura3Δ0 met15Δ0* (type II survivor)	This study

Telomeric DNA dot blots: DNA was purified using the Promega (Madison, WI) DNA purification kit and treated with RNaseA. DNA was then spotted onto a nylon membrane, X-linked with Stratalinker (Stratagene, La Jolla, CA), and incubated overnight at 50° with radiolabeled probes to specifically detect the G-rich or C-rich telomeric strands.

RESULTS

Overexpression of *PIF1* impairs cell growth: Several groups have reported that *PIF1* overexpression causes growth inhibition (Lahaye *et al.* 1991; Gelperin *et al.* 2005; Banerjee *et al.* 2006; Vega *et al.* 2007). The effect ranges from "moderate" to "strong," depending on whether the overexpression is driven from a galactose-inducible promoter on a low-copy centromeric plasmid (Vega *et al.* 2007) or from a galactose-inducible promoter on a high-copy 2 μm plasmid (Lahaye *et al.* 1991), respectively. Mild overexpression of *PIF1* under the control of its endogenous promoter from a 2μm plasmid yields no observable growth defect (Wagner *et al.* 2006). Thus, cell growth defects are augmented as Pif1 levels increase.

We also find that strong overexpression of *PIF1* dramatically impairs cell growth (Lahaye *et al.* 1991) (Figure 1A). The growth impairment is not associated with the mitochondrial function of Pif1, as it is still apparent in ρ^0 strains lacking mitochondrial DNA (Figure 1A). Furthermore, there is no increase in mitochondria-defective ρ^- cells following overexpression of *PIF1* (Lahaye *et al.* 1991). Moreover, moderate overexpression of an allele that expresses only the nuclear form of Pif1 also causes a mild growth defect (Vega *et al.* 2007). Therefore the growth impairment is due to the nuclear functions of Pif1.

Strong overexpression of *PIF1* activates a DNA damage response: To study why overexpression of *PIF1* impairs growth, we analyzed cell cycle progression by flow cytometry after the addition of galactose to induce expression of *PIF1* (Figure 1B). Strong overexpression of *PIF1* causes a modest accumulation of cells in S phase, which often results from the activation of replication checkpoints. Therefore, we assayed the activation of the Rad53 checkpoint kinase by analyzing both its phosphorylation-dependent mobility shift (data not shown) and its kinase activity. We find that Rad53 is robustly activated in cells strongly overexpressing *PIF1* (Figure 1C). Furthermore, moderate overexpression of *PIF1* in checkpoint-defective *rad53-11* mutants results in dramatic growth impairment (Figure 1D). Moderate overexpression of *PIF1* also renders cells sensitive to even low amounts of the replication inhibitor hydroxyurea (HU) and the DNA damaging agent methyl methanesulfonate (MMS) (Figure 1E). Taken together, our results indicate that the toxicity caused by *PIF1* overexpression at least partially results from DNA damage that activates the Rad53-dependent checkpoint pathway.

Rfa1 and Mre11 foci form upon strong overexpression of *PIF1*: Replication protein A (RPA), which consists of the subunits Rfa1, Rfa2, and Rfa3, binds single-stranded DNA (ssDNA) and is important for most aspects of eukaryotic DNA metabolism (Sakaguchi *et al.* 2009). RPA-coated ssDNA is a key structure for the activation of the DNA damage checkpoint response

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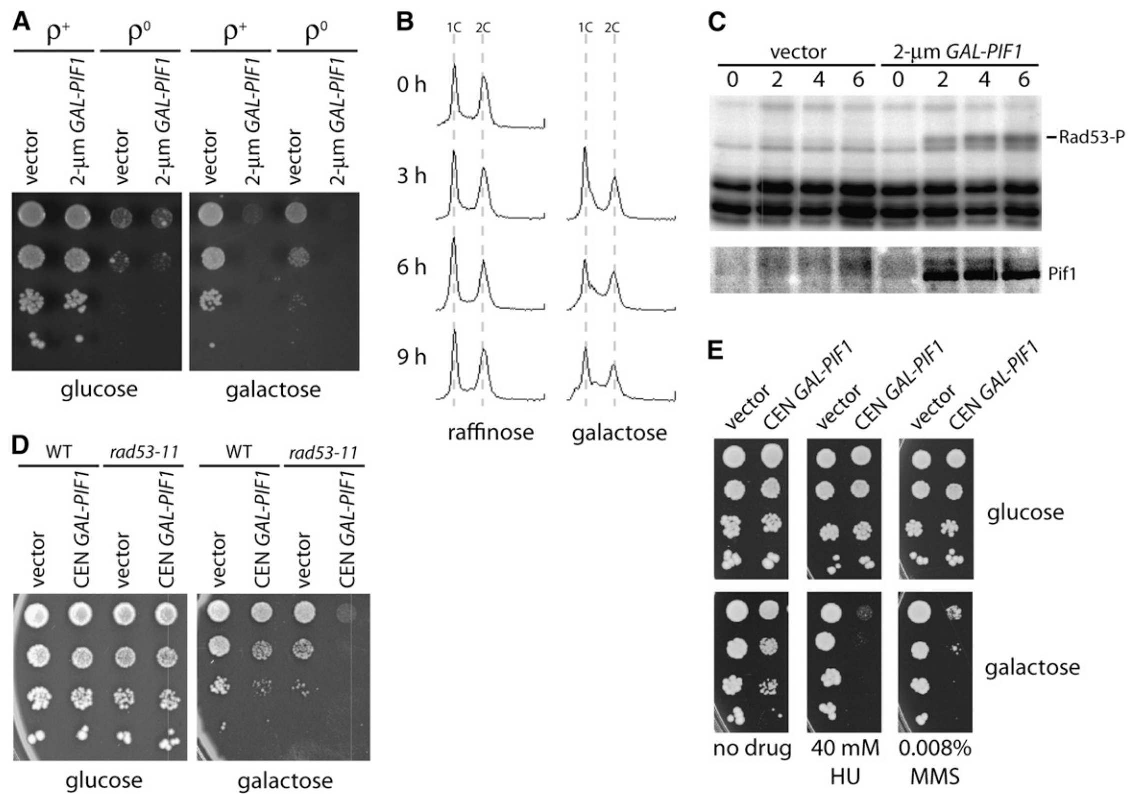

FIGURE 1.—*PIF1* overexpression impairs cell growth due to the accumulation of DNA damage. (A) ρ^+ and ρ^0 cells were transformed with either the 2μm *GAL-PIF1* plasmid (BG1805-PIF1) or a vector control (BG1766). Tenfold serial dilutions were then spotted onto either glucose or galactose media. (B) Wild-type cells containing BG1805-PIF1 were grown logarithmically in raffinose media. The culture was split in two and galactose was added to one to induce the overexpression of *PIF1*. Samples were removed at the indicated times after the addition of galactose and analyzed by flow cytometry. The positions of cells with 1C and 2C DNA content are indicated. (C) Wild-type cells transformed with the indicated plasmids were grown in raffinose-containing media. At the indicated number of hours after the addition of galactose, samples were fixed with TCA and extracts were fractionated by SDS–PAGE for *in situ* kinase assay of Rad53 (top panel). A parallel blot was probed to detect overproduced Pif1 (bottom panel). (D) Wild-type and *rad53-11* cells were transformed with either the centromeric *GAL-PIF1* plasmid (pVS45), which expresses the nuclear form of Pif1, or a vector control (pSH380). Tenfold serial dilutions were then spotted onto either glucose or galactose media. (E) Wild-type cells containing the indicated plasmids were spotted onto minimal media containing either 40 mM HU or 0.008% (v/v) MMS.

(Zou and Elledge 2003). Rfa1 forms nuclear foci following exposure to both ionizing radiation (IR), which generates DSBs, and HU, which stalls DNA replication fork progression by depleting dNTP pools (Lisby *et al.* 2004). We find that Rfa1 foci form upon strong overexpression of *PIF1* (Figure 2), indicating that the DNA damage caused by high levels of Pif1 induces the accumulation of ssDNA. Only a small percentage of Rfa1 foci colocalizes with Rap1 (Figure 2A), a protein found at telomeres (Klein *et al.* 1992; Gotta *et al.* 1996), indicating that the damage is not specifically localized to telomeres.

Mre11, Rad50, and Xrs2 form a complex that is required for NHEJ and homologous recombination and are among the first proteins to localize to a DSB (Krogh and Symington 2004; Lisby *et al.* 2004). We find that Mre11 also forms foci following strong overexpression of *PIF1* (Figure 2B), indicating the presence of DSBs. Seventy percent of Mre11 foci colocalize with Rfa1 foci, similar to the ~50% colocalization seen after IR treatment (Lisby *et al.* 2004). Furthermore, the increase in both Rfa1 and Mre11 foci is found exclusively in budded cells following strong overexpression of *PIF1* (Figure 2, bar graphs), indicating that the damage likely requires passage through S phase, consistent with known roles of Pif1 during S phase (Boule and Zakian 2006) and the S-phase delay induced by strong overexpression of *PIF1* (Figure 1B).

***PIF1* synthetic dosage lethality screen:** Synthetic dosage lethality (SDL) screens can identify functionally interacting genes and pathways (Kroll *et al.* 1996; Measday and Hieter 2002). To further characterize Pif1, we performed an SDL screen, searching for genes required for viability when *PIF1* is moderately overexpressed. We systematically introduced the centromeric plasmid containing galactose-inducible *PIF1* into the complete collection of ~4800 viable haploid gene deletion mutations, using SGA methodology (Tong *et al.* 2004; Tong and Boone 2006). Plasmid-containing mutants that showed reduced growth rates on galactose

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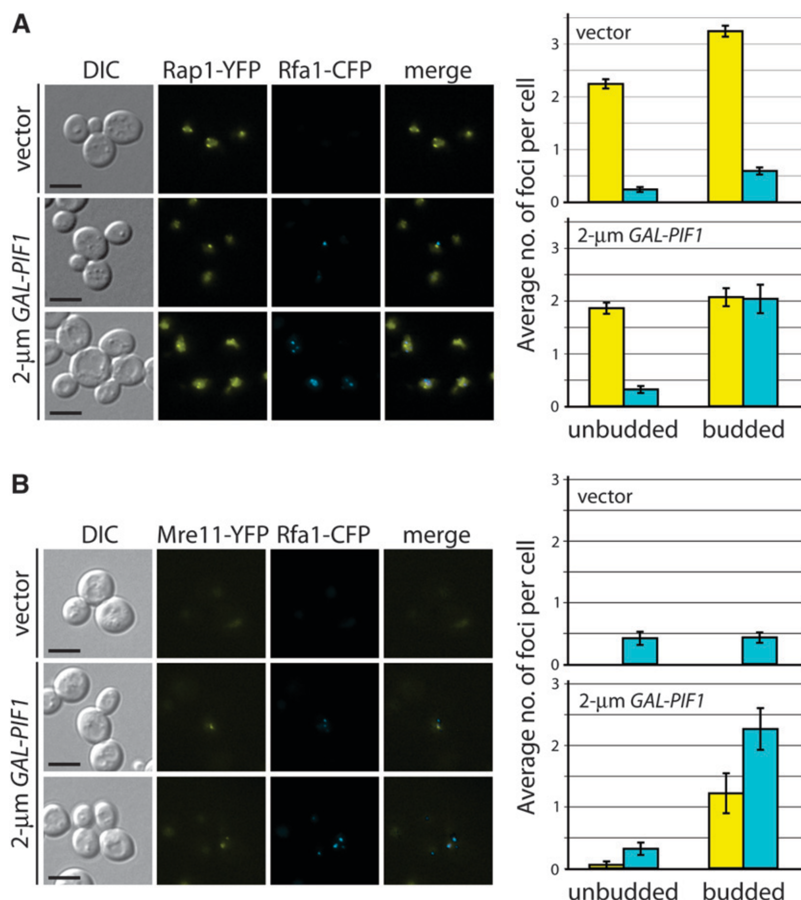

FIGURE 2.—Rfa1 and Mre11 form foci upon strong overexpression of *PIF1*. (A) Cells expressing Rap1-YFP and Rfa1-CFP, containing either a 2µm *GAL-PIF1* plasmid (BG1805-PIF1) or a vector control (BG1766), were grown in raffinose media followed by the addition of galactose. Images were acquired 6 hr after galactose addition. Scale bar, 5 µm. (B) As in A, except cells expressed Mre11-YFP instead of Rap1-YFP. Graphs show the average number per cell of Rap1-YFP or Mre11-YFP foci (yellow bars) and Rfa1-CFP foci (blue bars) in either unbudded or budded cells.

media were validated by reintroducing the *PIF1* plasmid or vector control into the gene deletion mutants by classical yeast transformation techniques, followed by serial spot dilutions onto media containing either glucose or galactose to assay for cell growth. Since the screen did not include essential genes, several viable mutations of essential genes were also tested for their sensitivity to moderate overexpression of *PIF1* and are included in Table 2. The list shows a strong enrichment for genes involved in DNA replication and the DNA damage response (*POL1*, *PRI2*, *CTF4*, *CDC2/POL3*, *POL32*, *DNA2*, *RAD27*, *ELG1*, *CDC9*, *MRC1*, *RAD9*, *SGS1*, *CTF18*, *DCC1*, *CTF8*, *ASF1*, and *RTT109*) (Table 3). Interestingly, most of the replication genes specifically affect lagging-strand synthesis. These results are consistent with overexpression of *PIF1* inducing DNA damage via interfering with lagging-strand DNA replication.

As expected from previous reports (Banerjee *et al.* 2006; Vega *et al.* 2007), we find that *yku70*Δ, *yku80*Δ, and *cdc13-1* mutants, all of which accumulate ssDNA at telomeres, are sensitive to moderate *PIF1* overexpression (Figure 3). Cdc13 interacts with Stn1 and Ten1 to form an RPA-like complex (Gao *et al.* 2007) that binds the telomeric G tail and prevents degradation of the C-rich strand (Garvik *et al.* 1995). We find that *stn1-13* mutants, which also have elevated levels of telomeric ssDNA (Grandin *et al.* 1997), are sensitive to moderate *PIF1* overexpression as well (Figure 3B).

To determine whether telomerase and/or nontelomerase functions of Pif1 are important for growth inhibition, we took advantage of the *est2-up34* allele, which encodes a telomerase catalytic subunit that is refractory to negative regulation by Pif1 (Eugster *et al.* 2006), and tested its ability to suppress the SDL interactions (Table 2). Interestingly, expression of *est2-up34* rescues the sensitivity of *yku70*Δ and *yku80*Δ, but not *cdc13-1* or *stn1-13*, to moderate overexpression of *PIF1* (Figure 3). Therefore the mechanism by which the Ku heterodimer caps telomeres is distinct from that of Cdc13 and Stn1. Indeed, Ku and Cdc13 define two different epistasis groups required for telomere maintenance (Nugent *et al.* 1998).

Telomerase is needed to repair Pif1-induced DNA damage: Deletions in genes encoding the protein subunits of telomerase (Est1, Est2, and Est3), while viable, could not be examined in the *PIF1* SDL screen because they senesce during the many growth selection steps involved in the SGA protocol used in this study. Hence, we tested a deletion of *EST2* directly and surprisingly found that it is sensitive to moderate *PIF1* overexpression (Figure 4A). This phenotype is rescued by expression of wild-type *EST2* from a plasmid, but not by a catalytically dead *est2-D670A* allele (Figure 4B).

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TABLE 2

***PIF1* SDL interactions**

Mutation	ORF	*PIF1* SDL[b]	*est2-up34*[c]	Cellular role
pol1-1[a]	YNL102W	SS	N	DNA replication and repair
pol12-100[a]	YBL035C	SSS	N	DNA replication and repair
pri2-1[a]	YKL045W	SSS	N	DNA replication and repair
*ctf4*Δ	YPR135W	SSS	N	DNA replication and repair
cdc2-2[a]	YDL102W	SS	N	DNA replication and repair
*pol32*Δ	YJR043C	SSS	N	DNA replication and repair
dna2-1[a]	YHR164C	SSS	N	DNA replication and repair
*rad27*Δ	YKL113C	SSS	N	DNA replication and repair
*elg1*Δ	YOR144C	S	N	DNA replication and repair
cdc9-1[a]	YDL164C	SSS	N	DNA replication and repair
*mrc1*Δ	YCL061C	SSS	N	DNA replication and repair
*rad9*Δ	YDR217C	S	N	DNA replication and repair
*sgs1*Δ	YMR190C	S	N	DNA replication and repair
rad53-11	YPL153C	SSS	Weak	DNA replication and repair
*ctf18*Δ	YMR078C	SSS	N	DNA replication and repair
*dcc1*Δ	YCL016C	SSS	N	DNA replication and repair
*ctf8*Δ	YHR191C	SSS	N	DNA replication and repair
*asf1*Δ	YJL115W	SS	N	DNA replication and repair
*rtt109*Δ	YLL002W	S	N	DNA replication and repair
*yku70*Δ	YMR284W	SSS	Y	Telomere maintenance
*yku80*Δ	YMR106C	SSS	Y	Telomere maintenance
cdc13-1[a]	YDL220C	SSS	N	Telomere maintenance
stn1-13[a]	YDR082W	SS	N	Telomere maintenance
*stm1*Δ	YLR150W	S	N	Telomere maintenance
*est2*Δ	YLR318W	SSS	Y	Telomere maintenance
*ctf19*Δ	YPL018W	SS	N	Kinetochore/spindle
*ctf3*Δ	YLR381W	SS	N	Kinetochore/spindle
*mcm16*Δ	YPR046W	SS	N	Kinetochore/spindle
*mcm22*Δ	YJR135C	SS	N	Kinetochore/spindle
*iml3*Δ	YBR107C	SS	N	Kinetochore/spindle
*chl4*Δ	YDR254W	SS	Weak	Kinetochore/spindle
*irc15*Δ	YPL017C	SS	N	Kinetochore/spindle
*ctk1*Δ	YKL139W	SS	N	Other
*mga2*Δ	YIR033W	S	N	Other
*nup84*Δ	YDL116W	S	N	Other
*rpa34*Δ	YJL148W	SS	N	Other
*rrp6*Δ	YOR001W	S	N	Other
*tsa1*Δ	YML028W	S	N	Other
*vps3*Δ	YDR495C	S	N	Other
*yaf9*Δ	YNL107W	SS	N	Other
*yke2*Δ	YLR200W	SS	N	Other

[a] SDL interaction was tested at 23°. All other SDL interactions were tested at 30°.
[b] SSS, strong sensitivity; SS, moderate sensitivity; S, mild sensitivity.
[c] Indicates whether the SDL interaction can be suppressed by the expression of the *est2-up34* allele. N, no; Y, yes.

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Thus *PIF1* overexpression is likely causing damage at telomeres and telomerase activity is required to repair this damage. The *est2-up34* allele, which fails to respond to Pif1 negative regulation, can also rescue the sensitivity of *est2*Δ. However, expression of this allele does not alleviate the mild toxicity associated with moderate overexpression of *PIF1* in either an *EST2* or an *est2*Δ genetic background (Figures 3 and 4C). This observation implies that while telomerase is likely required to repair Pif1-induced damage at telomeres, damage elsewhere in the genome requires other repair pathways.

Cells can propagate in the absence of telomerase by maintaining their telomeres via recombination-based mechanisms, and for yeast, these cells are called "survivors" (McEachern and Haber 2006). *S. cerevisiae* has two main recombination-mediated pathways that yield survivors: type I and type II. Type I survivors, which are Rad51 dependent, are characterized by the amplification of subtelomeric repeats while Rad50-dependent type II survivors are characterized by amplification of the TG-telomeric repeats. We tested whether activation of either pathway could suppress the sensitivity of telomerase-negative strains to moderate *PIF1* overex-

TABLE 3

Overrepresented Gene Ontology (GO) categories as determined by AmiGO (Carbon *et al.* 2009)

GO ID	GO biological process	Sample frequency (%)	Background frequency (%)	*P*-value
0006259	DNA metabolic process	25/41 (61)	433/6348 (6.8)	1.34×10^{-19}
0006974	Response to DNA damage stimulus	21/41 (51.2)	254/6348 (4.0)	2.57×10^{-19}
0006260	DNA replication	16/41 (39.0)	157/6348 (2.5)	5.50×10^{-16}
0006281	DNA repair	17/41 (41.5)	203/6348 (3.2)	1.51×10^{-15}
0034984	Cellular response to DNA damage stimulus	17/41 (41.5)	217/6348 (3.4)	4.67×10^{-15}
0006950	Response to stress	25/41 (61.0)	716/6348 (11.3)	2.43×10^{-14}
0051276	Chromosome organization	20/41 (48.8)	386/6348 (6.1)	2.44×10^{-14}
0006273	Lagging-strand elongation	8/41 (19.5)	20/6348 (0.3)	1.75×10^{-13}
0060249	Anatomical structure homoeostasis	11/41 (26.8)	68/6348 (1.1)	2.25×10^{-13}
0000723	Telomere mainenance	11/41 (26.8)	68/6348 (1.1)	2.25×10^{-13}
0032200	Telomere organization	11/41 (26.8)	68/6348 (1.1)	2.25×10^{-13}
0006261	DNA-dependent DNA replication	12/41 (29.3)	97/6348 (1.5)	4.43×10^{-13}
0007059	Chromosome segregation	13/41 (31.7)	137/6348 (2.2)	1.31×10^{-12}
0033554	Cellular response to stress	19/41 (46.3)	467/6348 (7.4)	1.10×10^{-11}
0051716	Cellular response to stimulus	19/41 (46.3)	483/6348 (7.6)	2.00×10^{-11}
0006271	DNA strand elongation during DNA replication	8/41 (19.5)	34/6348 (0.5)	2.36×10^{-11}
0022616	DNA strand elongation	8/41 (19.5)	34/6348 (0.5)	2.36×10^{-11}
0050896	Response to stimulus	25/41 (61.0)	1020/6348 (16.1)	8.23×10^{-11}

Biological processes that are enriched with a *P*-value $<1 \times 10^{-10}$ are shown.

pression. Since type I survivors are unstable and frequently convert to type II survivors, we tested type I survivors in a *rad50Δ* background, which prevents the formation of type II survivors (Figure 4D). We find that both type I and type II survivors fail to rescue the sensitivity of telomerase-negative strains to elevated levels of Pif1 (Figure 4, D and E). Thus, while recombination-based mechanisms exist to allow cells to propagate in the absence of telomerase, these mechanisms are unable to sufficiently repair the damage caused by *PIF1* overexpression. Consistent with this view, we find that cells lacking Rad52, a key player in DSB repair and homologous recombination that is necessary for the formation of both type I and type II survivors (McEachern and Haber 2006), are not sensitive to moderate overexpression of *PIF1* (data not shown).

Accumulation of ssDNA at telomeres in *PIF1* overexpressing cells: Our data suggest that *PIF1* overexpression is causing DNA damage at telomeres. However, following strong overexpression of *PIF1*, we observe that only a small percentage of Rfa1 foci colocalizes with Rap1 (Figure 2A). To determine whether damage is indeed occurring at telomeres, we assayed for telomeric ssDNA by probing dot-blotted genomic DNA with radiolabeled telomeric oligonucleotides. Strong overexpression of *PIF1* results in an increase in the amount of telomeric ssDNA when probing for the G-rich (TG) strand (Figure 5). Telomeric ssDNA also accumulates in a *yku70Δ* mutant (Gravel *et al.* 1998) and was included as a positive control. Interestingly, the increase in telomeric ssDNA is specific for the G-rich strand, because it is not seen when probing for the C-rich (CA) strand (Figure 5). Since the G-rich strand is always the template for lagging-strand DNA synthesis, it is likely that Pif1-induced damage is specifically affecting lagging-strand replication. This view is consistent with Pif1's proposed role in Okazaki fragment maturation (Ryu *et al.* 2004; Budd *et al.* 2006; Boule and Zakian 2007; Stith *et al.* 2008) and our SDL screen results that mutants defective in lagging-strand synthesis are sensitive to moderate overexpression of *PIF1* (Table 2).

DISCUSSION

Pif1 removes telomerase from telomeres and DNA DSBs (Schulz and Zakian 1994; Boule *et al.* 2005) and it has roles during DNA replication (Ivessa *et al.* 2000; Ryu *et al.* 2004; Budd *et al.* 2006; Boule and Zakian 2007; Rossi *et al.* 2008; Stith *et al.* 2008). In this study, we show that overexpression of *PIF1* inhibits cell growth in a dose-dependent manner. This growth inhibition is independent of Pif1's role in removing telomerase from DNA ends since neither preventing Pif1 from negatively regulating telomerase using the *est2-up34* allele (Figures 3 and 4C) nor removing telomerase by deleting *EST2* (Figure 4A) alleviates the toxicity. In addition, we find that lagging-strand replication mutants are sensitive to overexpression of *PIF1*. Therefore, we propose a model whereby *PIF1* overexpression causes DNA replication defects. At telomeres, these defects are repaired by telomerase activity.

Elevated levels of Pif1 interfere with DNA replication: Pif1 is found largely in the nucleolus where it associates with rDNA (Ivessa *et al.* 2000; Wagner *et al.* 2006). Pif1 helps maintain the replication fork barrier at the rDNA (Ivessa *et al.* 2000), suggesting that overexpression of *PIF1* may cause excessive levels of replication fork pausing.

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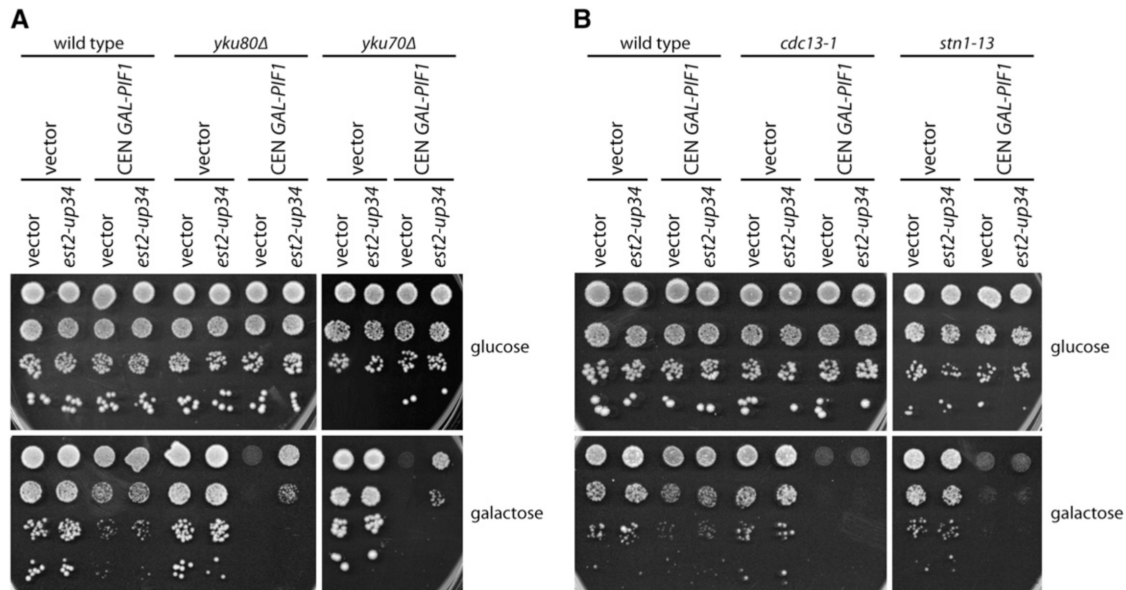

FIGURE 3.—*est2-up34* can rescue the sensitivity of *yku*Δ to moderate overexpression of *PIF1*. Tenfold serial dilutions were spotted onto media containing either glucose or galactose. (A) The indicated strains were transformed with either a vector control (pSH380) or the centromeric *GAL-PIF1* plasmid (pVS45), along with a second plasmid, either a vector control (pRS313) or a plasmid expressing *est2-up34*. (B) As in A, except strains were grown at 23° instead of 30°.

Pif1 also functions during the maturation of Okazaki fragments. Synthesis of these short stretches of DNA generated by lagging-strand synthesis is initiated by the DNA polymerase α-primase complex (Pol α-primase), leaving an RNA/DNA primer consisting of ∼10 nucleotides (nt) of RNA followed by 10–20 nt of DNA (Bambara *et al.* 1997; Liu *et al.* 2004). This primer is extended by DNA polymerase δ (Pol δ), in a complex with the proliferating cell nuclear antigen (PCNA) sliding clamp and the replication factor C (RFC) clamp loader, until it encounters the 5′ end of the downstream Okazaki fragment, which it can displace to produce a flap. This flap is cleaved by nucleases, leaving a nick that can be subsequently sealed by DNA ligase I (Garg and Burgers 2005).

The flaps are typically short and can be cleaved by the flap endonuclease Rad27/Fen1, but longer flaps ranging from 20–30 nt can be generated, which are then coated by RPA (Bae *et al.* 2001; Kao *et al.* 2004). These longer flaps require processing by the helicase/nuclease Dna2 before further cleavage by Rad27 (Bae *et al.* 2001; Kao *et al.* 2004). Since deletion of *DNA2* is lethal, but a *dna2*Δ *pif1*Δ double mutant is viable (Budd *et al.* 2006), it was proposed that Pif1 promotes the formation of long flaps that need to be processed by Dna2. Indeed, recent biochemical evidence shows that Pif1 accelerates long flap growth, allowing RPA to bind (Rossi *et al.* 2008). Our data support this model as we find that elevated levels of Pif1 cause Rfa1 foci formation (Figure 2A) and synthetic dosage lethality with *dna2-1* and *rad27*Δ (Table 2).

We also find that overexpression of *PIF1* is toxic to *cdc9-1*, *pol1-1*, *pol12-100*, *pri1-2*, *ctf4*Δ, *cdc2-2*, *pol32*Δ, and *elg1*Δ, all mutants with defects in lagging-strand synthesis (Table 2). *CDC9* encodes DNA ligase I (Johnston and Nasmyth 1978). *POL1* encodes the catalytic subunit of Pol α while *POL12* and *PRI1* encode additional subunits of Pol α (Plevani *et al.* 1988). Ctf4 physically interacts with Pol1 (Miles and Formosa 1992). *CDC2/POL3* encodes the catalytic subunit of the lagging-strand polymerase Pol δ (Blank and Loeb 1991). Pol32 is a subunit of Pol δ that is required for optimum processivity (Burgers and Gerik 1998; Gerik *et al.* 1998; Johansson *et al.* 2004). Elg1 interacts with Rfc2-5 to form an alternative RFC complex that has been proposed to function during Okazaki fragment maturation (Bellaoui *et al.* 2003; Kanellis *et al.* 2003). We did not detect any mutants that are defective in leading-strand replication in our SDL screen. To ensure that these were not merely false negatives in our screen, we directly tested *dpb3*Δ, *dpb4*Δ, and *pol2-12*—mutations in genes important for leading-strand synthesis—and find that none are sensitive to moderate *PIF1* overexpression (supporting information, Figure S1). Taken together, our studies strongly suggest that elevated levels of Pif1 disrupt lagging-strand DNA synthesis by causing excessive long flap formation during Okazaki fragment maturation.

The DNA replication problems caused by overexpression of *PIF1* are likely responsible for the activation of the Rad53 checkpoint kinase (Figure 1C) and for the requirement of DNA damage response genes for viability (*MRC1*, *RAD9*, *SGS1*, *CTF18*, *DCC1*, *CTF8*, *ASF1*, and *RTT109*) (Table 2). Mrc1 and Rad9 are both important for activating Rad53 in response to replication stress or DNA damage (Sun *et al.* 1998; Alcasabas *et al.* 2001). Mrc1 is also a component of the DNA replication

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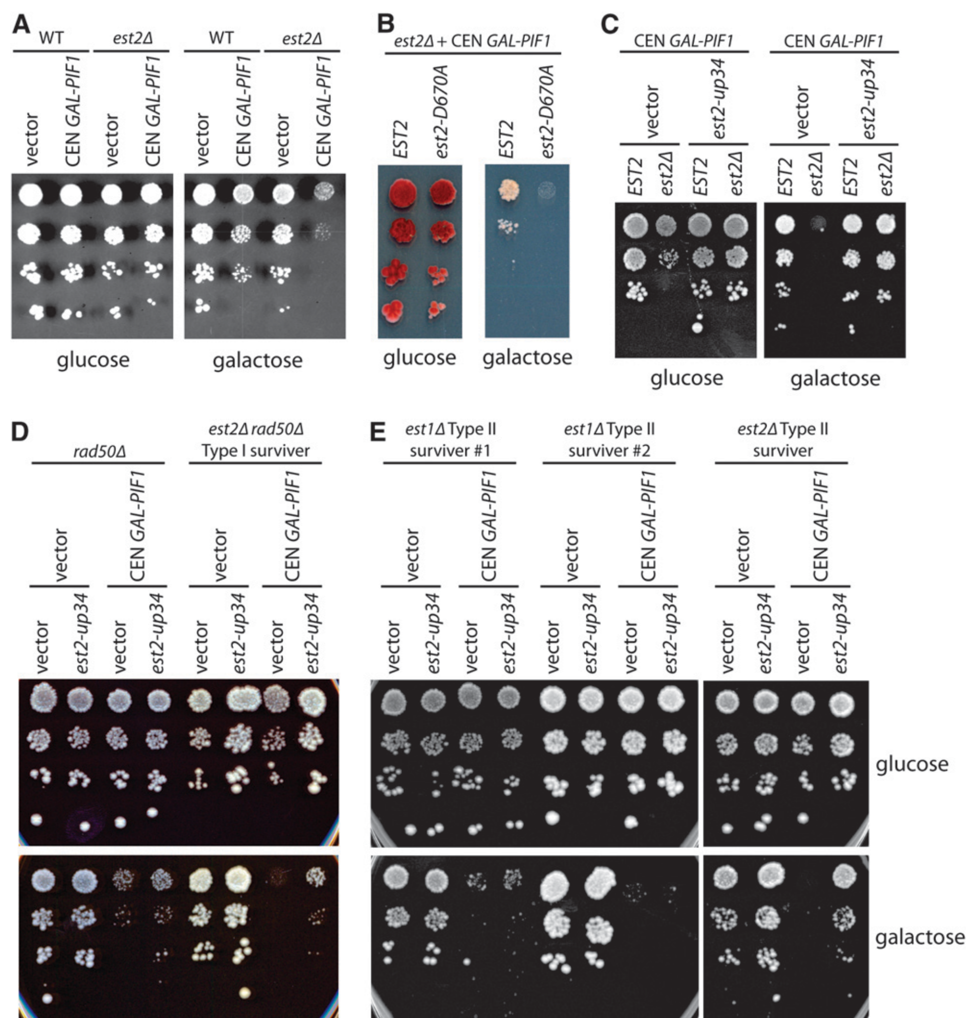

FIGURE 4.—Telomerase is needed to repair DNA damage caused by moderate overexpression of *PIF1*. Tenfold serial dilutions were spotted onto media containing either glucose or galactose. (A) An *EST2/est2*Δ heterozygous diploid was transformed with either a vector control (pSH380) or the centromeric *GAL-PIF1* plasmid (pVS45), sporulated, and tetrad dissected to obtain the indicated strains. (B) An *EST2/est2*Δ heterozygous diploid was transformed with pVS45 and a plasmid expressing either wild-type *EST2* or *est2-D670A*, sporulated, and tetrad dissected to obtain the indicated strains. (C) An *EST2/est2*Δ heterozygous diploid was transformed with pVS45 and either a vector control (pRS313) or a plasmid expressing *est2-up34*, sporulated, and tetrad dissected to obtain the indicated strains. (D and E) The indicated strains were transformed with either a vector control (pSH380) or the centromeric *GAL-PIF1* plasmid (pVS45), along with a second plasmid, either a vector control (pRS313) or a plasmid expressing *est2-up34*. As expected, survivors of *est2*Δ, but not *est1*Δ, senescence are rescued by the expression of the *est2-up34* allele.

machinery, moving along with the replication fork (Calzada *et al.* 2005; Szyjka *et al.* 2005; Tourriere *et al.* 2005). *SGS1* encodes a RecQ helicase that has important roles in maintaining genomic integrity (Bachrati and Hickson 2008; Bohr 2008). Pif1 counteracts Sgs1 helicase activity, preventing Sgs1-induced DNA damage that accumulates in a *top3*Δ mutant (Wagner *et al.* 2006). Thus overexpression of *PIF1* in a strain deleted for *SGS1* may greatly perturb the balance of opposing helicase activity, resulting in a loss of cell viability. Alternatively, Sgs1 may be involved in repairing damage caused by *PIF1* overexpression. Ctf18, Dcc1, and Ctf8, along with Rfc2-5, form an alternative RFC complex that has been linked to the DNA damage response and sister chromatid cohesion (Mayer *et al.* 2001; Naiki *et al.* 2001). Asf1 is a histone chaperone that functions with Rtt109, a histone acetyltransferase, to acetylate lysine K56 on histone H3 (Collins *et al.* 2007; Driscoll *et al.* 2007), which is critical for chromatin reassembly following DSB repair (Chen *et al.* 2008). Determining the interplay among these DNA damage factors will shed light on the cellular response to repair the lesion(s) caused by *PIF1* overexpression.

Kinetochore proteins are important for viability when *PIF1* is overexpressed: Intriguingly, we find that the deletion of several kinetochore genes renders cells sensitive to *PIF1* overexpression (Table 2). *CTF19*, *CTF3*, *MCM16*, *MCM22*, *CHL4*, and *IML3* encode proteins that function at the outer kinetochore (Measday *et al.* 2002; Pot *et al.* 2003). The outer kinetochore is thought to provide a link between the centromere-binding inner kinetochore proteins and microtubule-binding proteins. In addition, *IRC15,* which encodes a microtubule-associated protein that is important in establishing tension between sister kinetochores (Keyes and Burke 2009), was also identified in our screen. Replication forks pause at centromeres (Greenfeder and Newlon 1992) and this pausing is increased in the absence of Rrm3, a Pif1-like helicase (Ivessa *et al.* 2003). Although Pif1 and Rrm3 are ~40% identical and share many similar biochemical characteristics, they have largely nonoverlapping, often even opposing, functions (Boule and Zakian 2006). Thus overexpression of *PIF1*, like deletion of *RRM3*, may increase the pausing at centromeres and deleting kinetochore genes may exacerbate the phenotype. Our SDL results also suggest that some

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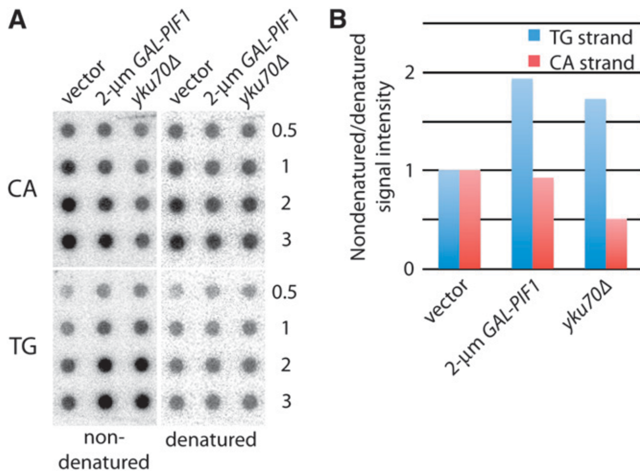

FIGURE 5.—Accumulation of telomeric ssDNA upon strong overexpression of *PIF1*. (A) A wild-type strain containing a vector control (BG1766), a wild-type strain containing the 2µm *GAL-PIF1* plasmid (BG1805-PIF1), and a *yku70*Δ strain were grown in galactose-containing media for 6 hr. DNA from these strains was digested with *Xho*I, which cuts at a site within the subtelomeric Y′ element. The DNA was analyzed by a non-denaturating DNA dot blot assay, using an oligonucleotide probe composed of either TG_{1-3} or $C_{1-3}A$ telomeric repeats, to probe for either the C-rich (CA) or the G-rich (TG) strand, respectively. Blots were then denatured and hybridized using the same telomeric probes to determine total telomeric DNA loaded. Numbers to the right of the blots indicate the amount (in micrograms) of total genomic DNA spotted. The experiment was also performed twice using an in-gel hybridization protocol (DIONNE and WELLINGER 1996), yielding similar results. (B) Quantification of the data in A. The data for each blot (TG or CA) were normalized to the wild-type strain containing the vector control.

kinetochore proteins may play important roles in promoting DNA replication through the centromeric region.

Consequences of *PIF1* overexpression at telomeres: We suggest that the problems associated with overexpressing *PIF1* are primarily caused by excessive Pif1 action during Okazaki fragment maturation. Accordingly, most of the *PIF1* SDL interactions are unaffected by the expression of the *est2-up34* allele (Table 2). However, the sensitivity of *yku*Δ mutations to moderate *PIF1* overexpression can be rescued by the *est2-up34* allele (Figure 3A), implying that in the absence of the Ku heterodimer, upregulation of Pif1's role as a negative regulator of telomerase is detrimental. Consistent with this view, *yku*Δ *est2*Δ double mutants exhibit accelerated senescence (NUGENT *et al.* 1998).

*yku*Δ mutants are unable to grow at 37° (FELDMANN and WINNACKER 1993; BOULTON and JACKSON 1996a,b; FELDMANN *et al.* 1996; BARNES and RIO 1997) and this temperature sensitivity can be rescued by increasing telomerase levels, either by overexpressing telomerase subunits (TEO and JACKSON 2001) or by mutating *PIF1* (VEGA *et al.* 2007; SMITH *et al.* 2008). However, neither the overexpression of *EST2* or *TLC1* nor the mutation of *PIF1* can suppress the short telomeres or elongated G tails of *yku*Δ cells (TEO and JACKSON 2001; SMITH *et al.* 2008). Interestingly, overexpression of *EST2* or *TLC1* suppresses activation of the Rad53 checkpoint kinase at 37° in *yku80*Δ cells (TEO and JACKSON 2001). Although it is not clear how telomerase antagonizes Rad53 activation, we suggest that increased levels of Pif1 kill *yku*Δ cells by removing telomerase from telomeres, causing activation of Rad53 and a checkpoint-mediated growth arrest.

In contrast to *yku*Δ mutants, expression of *est2-up34* cannot rescue the sensitivity of other telomere capping mutants such as *cdc13-1* and *stn1-13* (Figure 4B), indicating that the increased Pif1-mediated removal of telomerase from telomere ends is not responsible for the *PIF1* SDL interaction in these mutants. Cdc13 interacts with Pol1 (QI and ZAKIAN 2000; HSU *et al.* 2004) and Stn1 interacts with the Pol12 subunit of the Pol α-primase complex (GROSSI *et al.* 2004). Cdc13 and Stn1, along with Ten1, may have telomere-specific roles in DNA replication by acting as a telomere-specific RPA-like complex (GAO *et al.* 2007). Since we find that many replication mutants are sensitive to elevated levels of Pif1 (Table 2), we propose that *cdc13-1* and *stn1-13* may have replication defects at the telomere, resulting in sensitivity to overexpression of *PIF1*.

Another gene that is required for viability upon moderate overexpression of *PIF1* is *STM1*, which encodes a protein that can bind G quadruplexes (FRANTZ and GILBERT 1995), four-stranded DNA structures that form at highly G-rich sequences, such as those found at telomeres (JOHNSON *et al.* 2008). Stm1 physically interacts with Cdc13 and overexpression of *STM1* suppresses the temperature sensitivity of *cdc13-1* (HAYASHI and MURAKAMI 2002). Furthermore, Pif1 has been recently shown to unwind G quadruplexes (RIBEYRE *et al.* 2009), strengthening a possible link between G-quadruplex formation and telomere capping. Lack of Stm1 may weaken telomere capping and cause sensitivity to *PIF1* overexpression. However, Stm1 also has functions in mRNA decay and protein synthesis (VAN DYKE *et al.* 2006; BALAGOPAL and PARKER 2009) so it may protect cells from damage induced by *PIF1* overexpression through other mechanisms not related to telomere maintenance.

Telomerase is needed to repair Pif1-induced DNA damage at telomeres: Overexpression of *PIF1* causes accumulation of telomeric ssDNA (Figure 5), likely due to its interference with DNA replication at telomeres. The presence of Mre11 foci upon strong overexpression of *PIF1* (Figure 2B) indicates that the disruption of DNA replication can cause DSBs, possibly due to replication fork collapse. We propose that telomeric DSBs caused by elevated levels of Pif1 result in critically short, truncated telomeres requiring immediate elongation by telomerase to avoid telomere uncapping and cell death. Consistent with this model, we find that telomerase activity

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is required for viability upon overexpression of *PIF1* (Figure 4). In addition, our previous work has shown that *TEL1* is needed for the enhanced repeat addition processivity of telomerase necessary to elongate critically short telomeres (Chang *et al.* 2007). However, *TEL1* is not required for viability when *PIF1* is overexpressed (Vega *et al.* 2007). Furthermore, since deletion of *TEL1* also greatly reduces the frequency of telomere elongation (Arneric and Lingner 2007) and the association of telomerase with telomeres (Bianchi and Shore 2007; Hector *et al.* 2007; Sabourin *et al.* 2007), only a small amount of telomerase is sufficient to maintain viability upon moderate overexpression of *PIF1*. Nonetheless, this low level of telomerase is critical to repair damage created by elevated levels of Pif1.

In summary, our work shows the importance of carefully regulating the protein levels of Pif1 in the cell. We propose that overexpression of *PIF1* impairs lagging-strand synthesis, resulting in DNA damage. At telomeres, telomerase activity is needed to repair this damage. These studies reveal an important link between telomere replication and telomerase action that is mediated by the Pif1 helicase.

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Telomerase Is Essential to Alleviate Pif1-Induced Replication Stress at Telomeres

Michael Chang, Brian Luke, Claudine Kraft, Zhijian Lu, Matthias Peter,
Joachim Lingner and Rodney Rothstein

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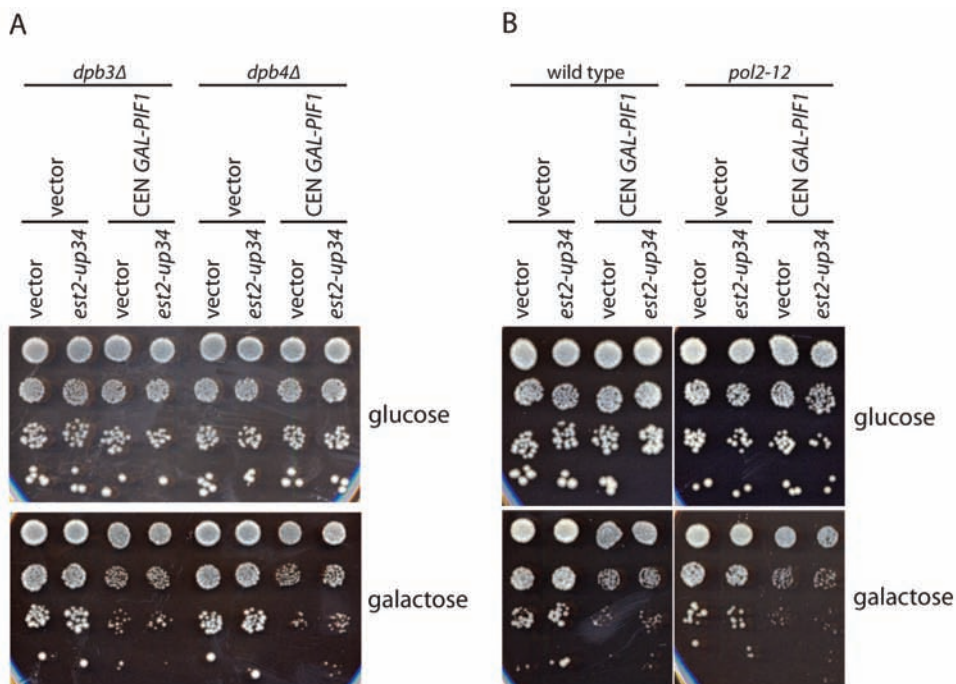

FIGURE S1.—Leading strand replication mutants are not sensitive to moderate overexpression of *PIF1*. In each panel, 10-fold serial dilutions were spotted onto media containing either glucose or galactose. (A) The indicated strains were transformed with either a vector control (pSH380) or the centromeric *GAL-PIF1* plasmid (pVS45), along with a second plasmid, either a vector control (pRS313) or a plasmid expressing *est2-up34*. (B) As in A, except strains were grown at 23°C instead of 30°C.

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