Supplementary Materials and Methods

Supplemental Table 1. *S. cerevisiae* strains used in this study

Strains	Genotype	Reference
W303	*MAT**a**/MAT**α** leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11, 15 RAD5+*	(1)
YCG59	W303 *PIF1/pif1::NatMX6 DNA2/dna2::KanMX6*	This study
YPH500	*MAT**α** ura3-52 lys2-801_amber ade2-101_ochre trp1Δ63 his3Δ200 leu2Δ1*	(2)
MBY77	YPH500 *hxt13::URA3 pif1::His3MX6*	M. Bochman

Supplemental Table 2. Plasmids used in this study

Name	Plasmid information	Reference
pVS102	CEN ARS *TRP1 PIF1* promoter *PIF1*	(3)
pMB13	CEN ARS *TRP1*	M. Bochman
pMB282	pMB13 *RRM3* promoter *PIF1* C-terminal 3xFLAG	(4)
pCG17	pMB282 *PIF1 promoter PIF1 C-terminal 3xFLAG*	This study
pCG18	pCG17-Pif1-K264A	This study
pCG19	pCG17-Pif1-SMΔ6	This study
pCG20	pCG17-Pif1- SMΔ21	This study
pCG22	pCG17-Pif1-P367A	This study
pCG23	pCG17-Pif1-L354P	This study
pCG24	pCG17-Pif1-F368A	This study
pCG25	pCG17-Pif1-G369A	This study
pCG26	pCG17-Pif1-I371A	This study
pCG27	pCG17-Pif1-G370A	This study
pCG30	pCG17-Pif1-L354A	This study
pCG41	pCG17-Pif1-SM6A	This study
pCG45	pCG17-Pif1-Q372A	This study
pCG46	pCG17-Dda α-helix	This study
pCG47	pCG17-Pif1-SM6G	This study
pCG48	pCG17-BsPif1-SM	This study
pCG50	pCG17-Pif1-SM15A	This study

Supplemental Figure 1. (A) Sequence alignment of Pif1-family helicases and T4 phage Dda. **(B)** Structural alignment of homology model of ScPif1 and T4 phage Dda helicase (PDB: 3upu). ScPif1 and Dda are both SFI DNA helicases. In ScPif1, the SM is located between helicase motifs II and III and folds into an α-helix with an extended loop. Structural analyses (5) show that the Dda helicase has α-helix and loop located between helicase motifs II and III. In the Dda α-helix allele, the sequence of the Dda helix replaces that of the ScPif1 α-helix.

Supplemental Figure 2. (A) Western blot analysis of 3xFLAG tagged WT and ScPif1 mutant proteins after resolution on a 5% SDS-PAGE gel as described in Materials and Methods. The protein expression was quantified by normalizing to the signal of the tubulin loading control. **(B)** Growth rate of WT and SM mutants grown in minimal media. Saturated cells were diluted to a starting OD660 of 0.05 and incubated at 30°C. The OD was measured hourly for up to ten hours and then the doubling time was calculated.

Supplemental Figure 3. Analysis of telomere lengths in cells expressing different *PIF1* alleles. Genomic DNA was isolated from three or more independent isolates. **(A)** Lanes contain DNA from WT (lanes 1, 16), *pif1Δ* (lanes 2, 15), Q372A (lanes 3-5), SM6G (lanes 6-8), BsPif1-SM (lanes 9-11), and Dda α-helix (lanes 12-14). **(B)** Lanes contain DNA from SM6A (lanes 1-3), F368A (lanes 4-6), G370A (lanes 7-9), and I371A (lanes 10-12). **(C)** Lanes contain DNA from WT (lanes 1-3), SMΔ21 (lanes 4-6), SMΔ6 (lanes 7-9), SM15A (lanes 10-12), P367A (lanes 12-15), and G369A (lanes 16-18). **(D)** Lanes contain DNA from WT (lanes 1, 14), K264A (lanes 2-4), L354A (lanes 5-7), F368A (lanes 8-10), and G370A (lanes 11-13). Average telomere lengths measured from these telomere blots were used to generate the graph in Figure 4B.

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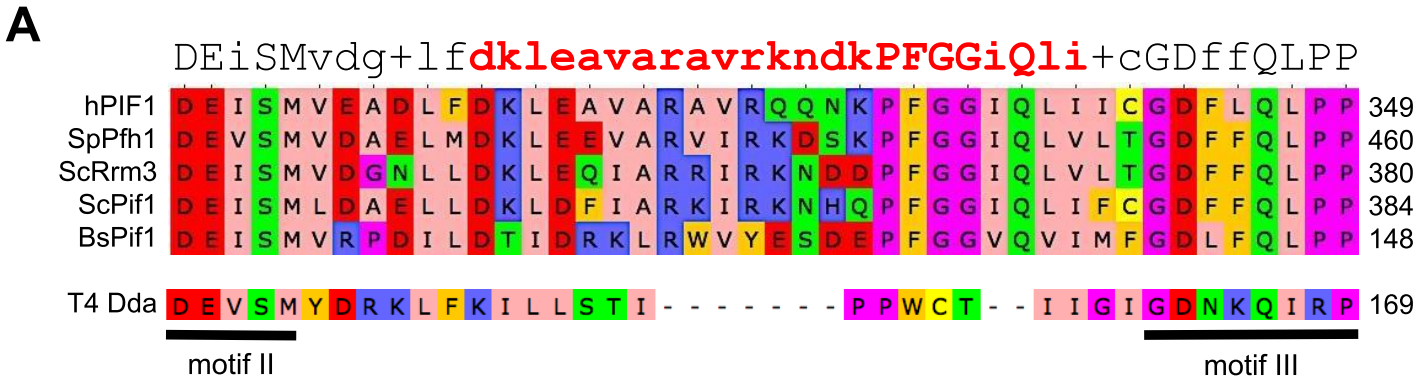
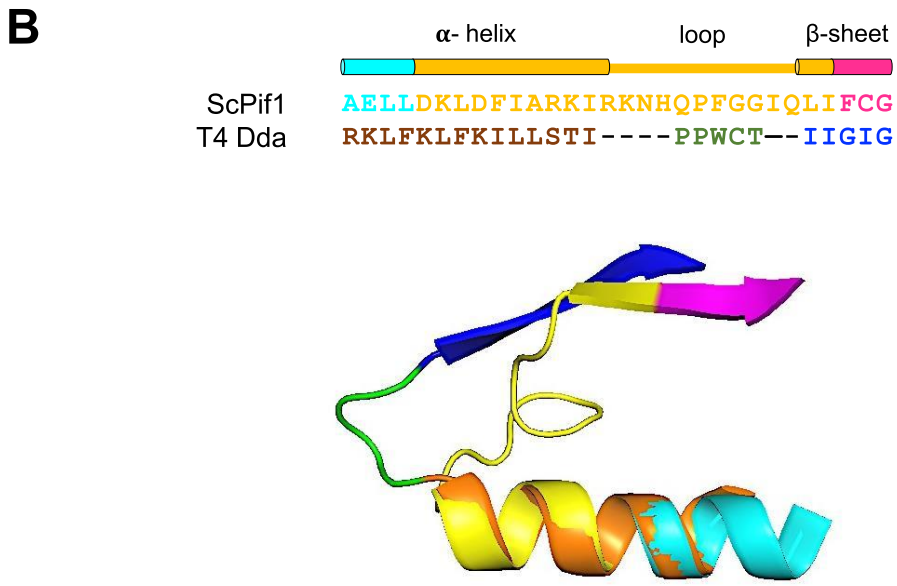
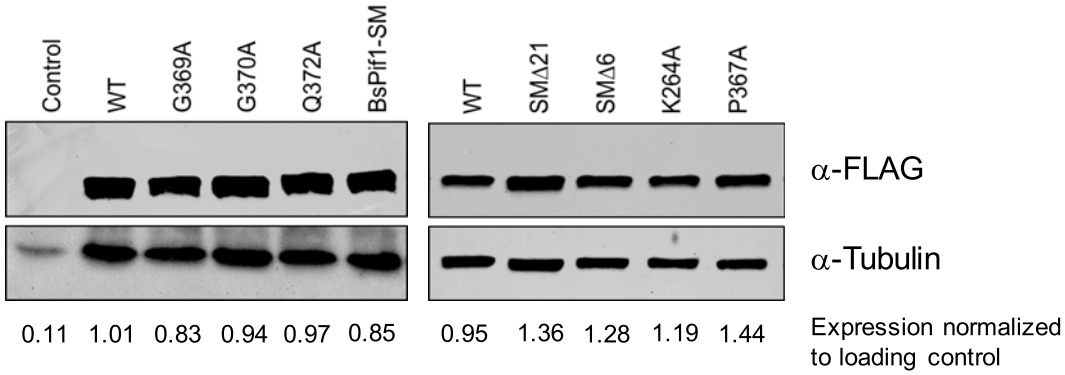
Supplemental Figure 1





Supplemental Figure 2

A



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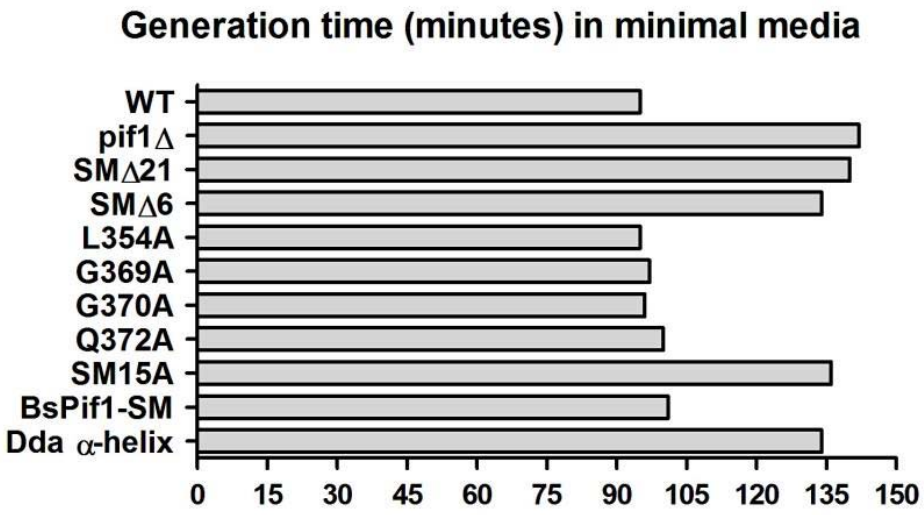
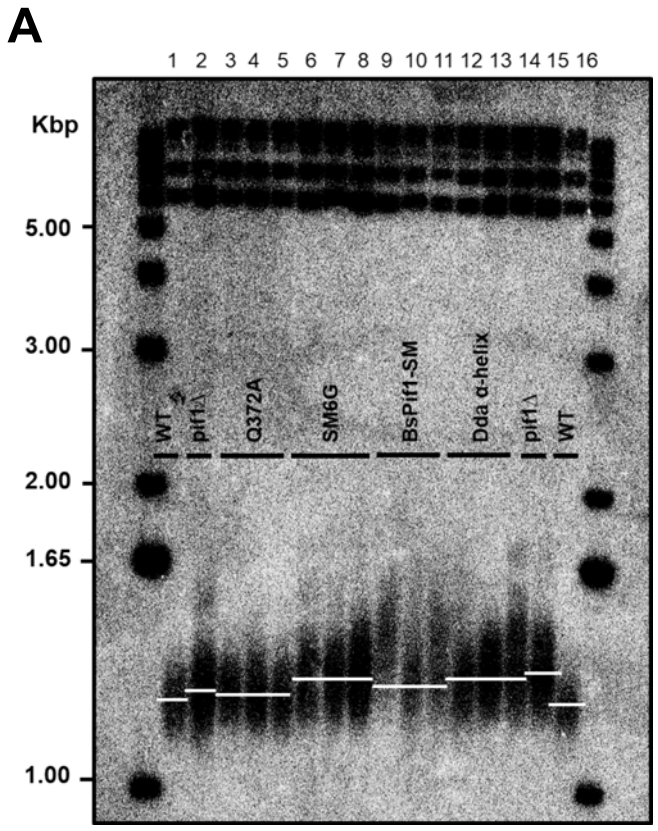
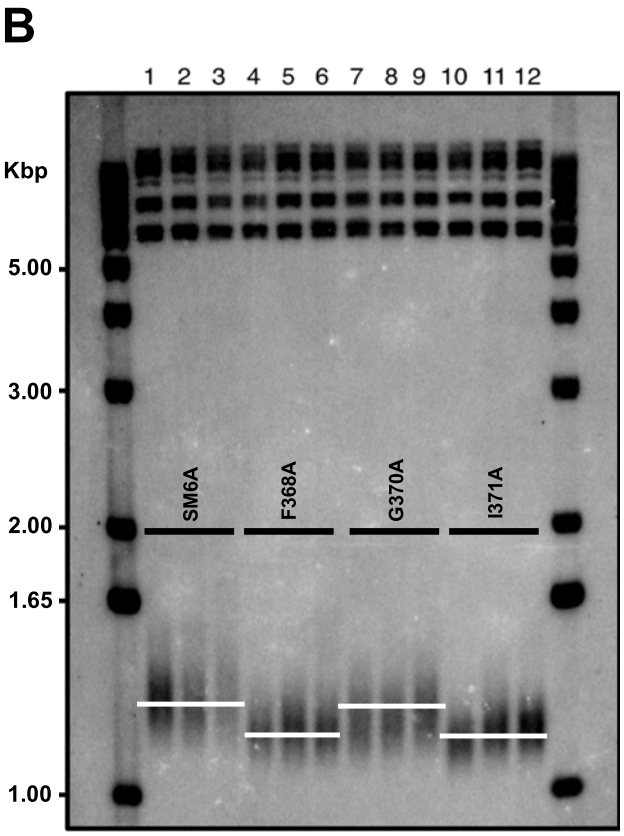
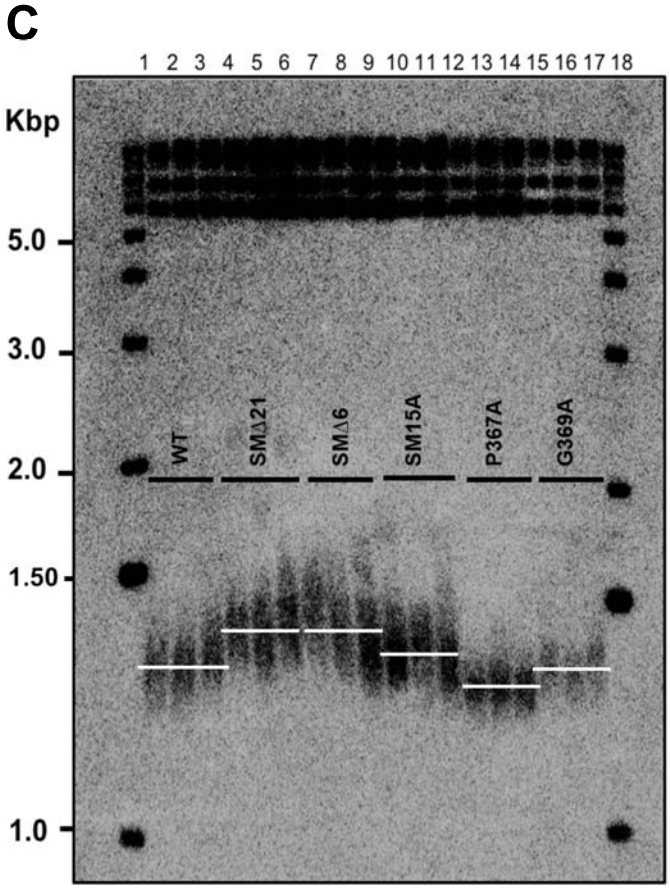
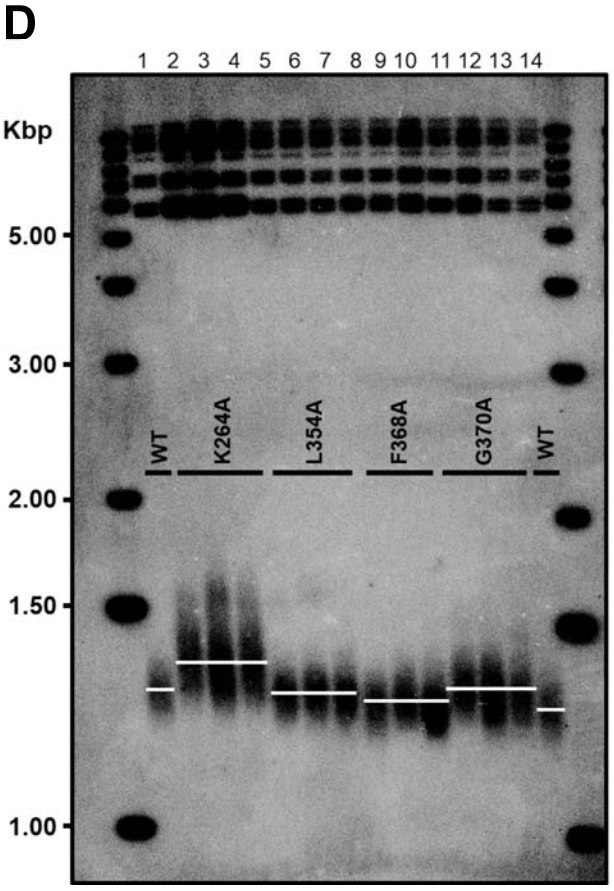


Supplemental Figure 3









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