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

References and Notes

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8. *Saccharomyces cerevisiae* strains isogenic to VPS106 were used (Fig. 1) (*2*). *RAD52* was inactivated by transforming cells with a fragment containing *LEU2* inserted at the unique Bgl II site within the coding region of *RAD52*. The *TLC1* gene was inactivated by transforming diploid VPS107 *PIF1/pif1-m2* cells with Xho I–digested pBlue61::LEU2, which deletes 693 bp from the *TLC1* coding region (*6*). The resulting diploid strain, VPS107*PIF1/pif1-m2 TLC1/tlc1::LEU2*, was sporulated, and tetrads were dissected. Individual spore products from nine tetrads were streaked four times on rich medium and examined at each streak for growth rate and viable colonies. DNA for analysis of telomere length was prepared ~30 cell divisions after sporulation and ~5 cell divisions after each restreak.
9. J.-Q. Zhou *et al.*, data not shown.
10. Mutagenesis of lysine 264 in *PIF1* to alanine (K264A) or arginine (K264R) was done using the QuickChange Site-directed Mutagenesis Kit (Stratagene) on the 488-bp Afl II–Pfl MI *PIF1* fragment, which was sequenced after mutagenesis to ensure that it contained only the desired mutation. The mutagenized fragment was inserted into pVS102, which contains a genomic Sac I–Apa I *PIF1* fragment, replacing the wild-type portion of the gene. The pVS102 mutant or wild-type *PIF1* plasmids were digested with Sac I–Cla I, and the fragment containing *PIF1* was inserted at the Bam HI site in the pFastBac1 vector (Gibco–BRL). For the purification of recombinant proteins, 1 liter of Sf9 cells at a density of 1.5×10^6/ml were infected at a multiplicity of 10 and harvested 60 hours after infection. The recombinant proteins in the cytoplasmic fraction were followed by Western blotting, using a Pif1p antiserum (*3*), and purified with chromatography on phosphor-cellulose, Q Sepharose, and heparin agarose.
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12. Experiments to assess the effects of eliminating the helicase activity of Pif1p were carried out in strain YPH499 (*28*). The *pif1*Δ allele deleted 1465 bp, extending from the first initiation codon of *PIF1* through amino acid 488. The K264A and K264R *PIF1* alleles were introduced by intergrative transformation in such a way as to replace the endogenous *PIF1*. The presence of point mutations was confirmed by DNA sequencing on PCR-amplified DNA. Pif1p was overproduced in YPH499 cells by inserting a Sac I–Apa I *PIF1* fragment that contained the *PIF1* ORF as well as 1291 bp 5′ and 2788 bp 3′ of the ORF into Sma I–digested YEpFAT7 to yield pVS41. Chromatin immunoprecipitations were done in the YPH499 background.
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20. The *S. pombe rph1*$^+$ was isolated using PCR primers that were based on conserved amino acid residues present in both *PIF1* and the *C. maltosa* Pif1p-like protein (accession number AF074944). By comparing the Pif1p sequence with the translated database of expressed sequence tags, we found one human gene (accession number AA464521) with low levels of similarity (expectation value of 0.015). We extended this sequence 3′ to the poly(A) tail by further searching of the human gene against the expressed sequence tag database (unigene number Hs.112160). The raw sequence was corrected by inspection of the automated sequencing traces, and when this sequence was compared to the protein database, high levels of similarity to the *PIF1* subfamily were found. We extended the sequence deduced from the database to 374 amino acids (accession number AF108138) using rapid amplification of cDNA ends. We were unable to obtain the sequence of the NH_2-terminal portion of hPif1 because, after 374 amino acids, its cDNA was fused to an Alu element as a result of template switching during cDNA synthesis.
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Pol κ: A DNA Polymerase Required for Sister Chromatid Cohesion

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

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

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

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

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DNA polymerase activity observed is dependent on template, primer, and Mg^{2+}. To ensure that the activity observed was not due to *E. coli* pol I contamination [the major polymerase activity in *E. coli* extracts (*9*)], we used a neutralizing monoclonal antibody to DNA pol I in the polymerase reactions (*10*). Figure 1C shows that incubation of the neutralizing antibody with DNA pol I inhibited its ability to extend the oligo(dT) primer, whereas a different monoclonal antibody to DNA pol I that does not neutralize the activity had no effect on DNA pol I activity (*10*). In contrast, Trf4 activity was unaffected by either monoclonal antibody (Fig. 1C, lanes 4 to 6). In addition, the size range of the Trf4 products was consistently observed to be greater than for DNA pol I (Fig. 1C).

Sensitivity of the Trf4 DNA polymerase to a variety of inhibitors used to classify DNA polymerases was determined (*11*, *12*). The Trf4 activity was resistant to aphidicolin (50 μg/ml) and to preincubation with the sulfhydryl-blocking agent *N*-ethylmaleimide (NEM; Fig. 2A). However, in the presence of equimolar amounts of dideoxythymidine triphosphate (ddTTP) and dTTP, the Trf4 activity was inhibited (Fig. 2A). Resistance to aphidicolin and NEM and sensitivity to dideoxynucleotide inhibition are properties of β-DNA polymerases (*11*, *13*). Furthermore, *E. coli* DNA pol II and pol III are sensitive to NEM and pol II is sensitive to aphidicolin (*9*), excluding contamination by these proteins as the source of the observed activity.

Trf4 is also able to use a complex DNA template (Fig. 2B). The oligo/template pairs, which differ in length by 1 nt at the 3′ end, were hybridized separately to the same 75 mer template oligonucleotide after the oligo primer was 5′ end-labeled with ^{32}P. Template/primer pairs were then incubated with Trf4 in the presence of all four dNTPs, and the products were analyzed on a urea-acrylamide gel. The polymerization reactions in each of these primer/template pairs were relatively processive, because by far the most abundant extension product is the approximate size of the template. This is in contrast to the distributive polymerization observed in the oligo(dT)/poly(dA) assay.

To determine whether Trf4 DNA polymerase activity is template directed, we hybridized the oligo primers used in Fig. 2B separately to the same 75 mer template oligo. The various primer/template junctions formed are illustrated in Fig. 2C. Extension of template/primer pair 1 by Trf4 is observed only in the presence of deoxycytidine triphosphate (dCTP), indicating that the polymerization reaction is not only template-dependent but also template-directed. Similarly, pair 2 is extended only in the presence of deoxyguanosine triphosphate (dGTP), pair 3 only with dTTP, and pair 4 only with deoxyadenosine triphosphate (dATP), demonstrating that all four deoxynucleotides are recognized by Trf4 and used as substrates for DNA synthesis in a template-directed manner.

Our previous genetic studies with *TRF4* indicate that it is required for mitotic chromosome segregation (*1*–*4*). One link between DNA replication and chromosome segregation is the establishment of cohesion between sister chromatids, which occurs during DNA replication (*14*–*16*) and is crucial for accurate segregation at anaphase. Furthermore, if DNA replication occurs in the absence of the cohesin *SCC1/MCD1*, expression of *SCC1/MCD1* after replication is insufficient to establish cohesion (*14*), suggesting a direct coupling of replication to cohesion. We have previously observed that *TRF4* displays both genetic and physical interactions with Smc1 (*2*), a protein required for sister chromatid cohesion (*17*, *18*).

To determine whether *TRF4* is required for sister chromatid cohesion, we monitored the state of cohesion in otherwise isogenic *TRF4*$^+$ and *trf4* mutant cells with two different assays. In the first assay, fluorescence in situ hybridization (FISH) of a biotin-labeled cosmid DNA probe located 225 kb from the centromere of chromosome XVI ("*CEN* distal" probe in Table 1) was used to examine sister chromatid cohesion (*19*). In yeast cells in which cohesion is effectively maintained, a single fluorescence signal is observed because of the close proximity of the sister chromatids (*19*). Cells were first arrested with the microtubule-depolymerizing drug nocodazole, which blocks the cell cycle in mitosis after the generation of sister chromatids, but before cohesion is dissolved during anaphase. Wild-type cells are known to maintain sister chromatid cohesion at the nocodazole block (*19*). FISH was then performed on over 50 nuclei from both wild-type and the *trf4* mutant.

As expected, the wild-type haploid strain showed only one hybridization signal in the majority of nuclei (87%), indicating that sister cohesion was intact (Fig. 3A and Table 1). The 13% of wild-type nuclei that display two or more signals has also been observed by others (*16*, *19*, *20*) and may represent partial disruption of cohesion by the fixation procedure. In contrast, the *trf4* mutant cells showed two hybridization signals in 41% of nuclei, demonstrating that cohesion is frequently defective in the absence of *TRF4* function. Simultaneous 4′,6′-diamidino-2-phenylindole (DAPI) staining was used to confirm that the

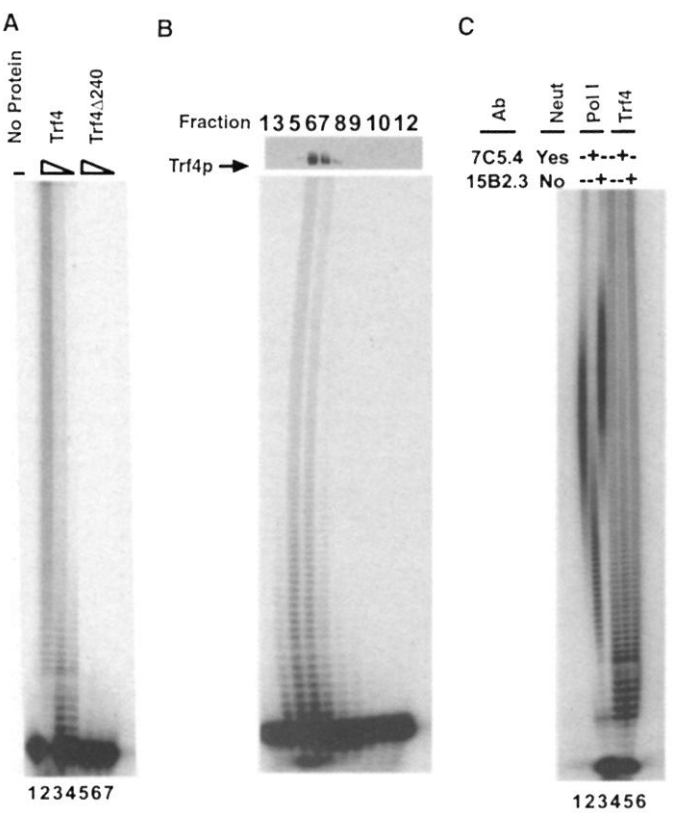

Fig. 1. (**A**) DNA polymerase assays with an oligo(dT)/poly(dA) primer/template. Equal amounts of purified recombinant Trf4-6HIS or Trf4Δ240-6HIS were incubated with 100 nM oligo(dT)/poly(dA) [20:1 molar excess of poly(dA):oligo(dT)] for 5 min at 37°C in 50 mM tris-HCl (pH 8.0) (pH at 22°C), 10 mM $MgCl_2$, 2 mM DTT, 20 mM NaCl, 20 mM KCl, 2.5% glycerol, bovine serum albumin (0.2 mg/ml), and 1 mM dTTP. No protein was added to lane 1. The concentration of both Trf4 and Trf4Δ240 is 150 nM in lanes 2 and 5, 15 nM in lanes 3 and 6, and 1.5 nM in lanes 4 and 7. (**B**) Cofractionation of Trf4 protein and DNA polymerase activity on a mono Q anion exchange column. DNA polymerase assays were performed as in (A). The top panel shows a Coomassie-stained SDS–polyacrylamide electrophoresis gel of the same column fractions. Immunoblots confirm the identify of the protein as Trf4. (**C**) Trf4 DNA polymerase activity is not sensitive to a neutralizing monoclonal antibody to DNA pol I. DNA polymerase assays were performed as in (A) in a total volume of 20 μl. Lanes 1 and 4 contain no added antibody, assays shown in lanes 2 and 5 contain 1 μg of DNA pol I neutralizing monoclonal antibody 7C5.4, and assays shown in lanes 3 and 6 contain 1 μg of DNA pol I nonneutralizing monoclonal antibody 15B2.3.

two fluorescence signals observed in the *trf4* mutant were derived from a single nucleus (Fig. 3A). FISH assays of a known cohesion protein, *MCD1/SCC1* (*20*), show a similar fraction (50 to 60%) of nuclei that fail to maintain cohesion under these conditions (*20*). The two hybridization signals that are observed in *trf4* mutant nuclei are the result of sister cohesion failure and are not due to the presence of two copies of chromosome XVI (*7*).

To examine sister cohesion at other chromosomal regions, we used a 35-kb centromere-proximal cosmid probe that spans a region between 11 and 45 kb from the centromere on the left arm of chromosome XVI ("*CEN* proximal" probe in Table 1). A similar cohesion defect is observed in the *trf4* mutant cells with this probe [Table 1 (*7*)], indicating that *TRF4* is required for cohesion both near centromeres and on chromosome arms.

We also used a second assay to monitor the cohesion status of a different chromosome. In the "green fluorescence protein (GFP)–marked chromosome" assay (*21*), chromosomes are visualized with immunofluorescence microscopy by the *GFP* fluorescence signal generated when a *GFP-lac* repressor fusion is expressed in a yeast strain bearing 256 tandem repeats of the *lac* operator integrated on one of the chromosomes (*21*). Of the over 100 nuclei examined for each data point in these experiments, two signals were present in only 2.5% of wild-type nuclei (Table 2). In contrast, the *trf4* mutant showed two signals in 18% of cells [Table 2 (*7*)], seven times more frequently than in wild type. Furthermore, the cohesion defect in *trf4* is anaphase promoting complex–independent because it is still observed in a *cdc16* mutant strain [Table 2 (*7*)], demonstrating that failure of the spindle assembly checkpoint is not the reason for its cohesion defect. Cohesion occurs at multiple distinct sites along each chromosome, with a concentration of sites near the centromere (*22*). Thus, chromosomes with defective cohesion at some sites and not others are likely to segregate normally. Therefore, it may not be surprising that the *trf4* single mutant remains viable. Consistent with this explanation, we observed that the *mcd1-1 ts* cohesion mutant also shows a clear cohesion defect even at the permissive temperature of 30°C (*7*). Thus, *TRF4* is required for sister chromatid cohesion as determined by two independent assays, on both chromosomes XVI and III and at both centromere-proximal and -distal loci.

To determine whether *TRF* function was required to maintain cohesion, we examined cohesion in the *trf4-ts trf5* double mutant. If *TRF* function were required to maintain cohesion, then cells arrested with nocodazole at a permissive temperature and then shifted to the nonpermissive temperature should show an increased cohesion defect. After nocodazole arrest at the permissive temperature of 30°C, the *trf4-ts trf5* double mutant was able to maintain cohesion as monitored by the GFP-marked chromosome assay. However, when mutant cells were shifted to 37°C for 3 hours, a marked increase in cohesion-defective cells was observed (Fig. 3B). This demonstrates that *TRF* function is indeed required to maintain cohesion.

The ability of cells lacking *TRF* function to duplicate the genome was then examined as follows. Synchronous populations of either wild-type or otherwise isogenic *trf4-ts trf5* double-mutant G_1 daughter cells were obtained by centrifugal elutriation. Wild-type and mutant cells were then released into yeast extract, peptone, and dextrose (YPD) medium at 37°C, and DNA content, budding index, and cell viability were monitored during a single synchronous cell cycle. Figure 3C shows that wild-type cells begin DNA syn-

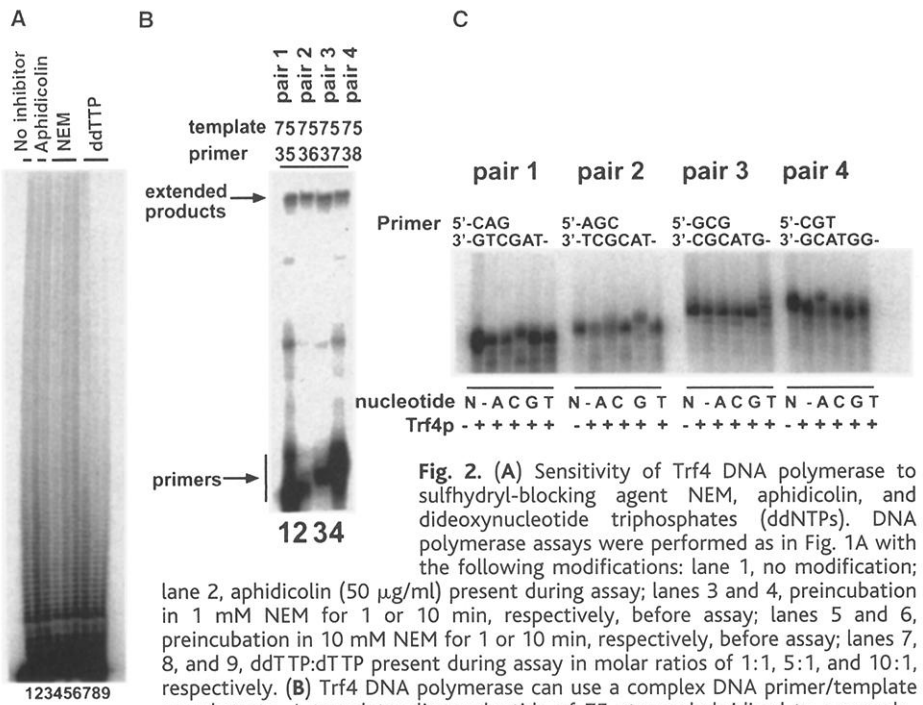

Fig. 2. (**A**) Sensitivity of Trf4 DNA polymerase to sulfhydryl-blocking agent NEM, aphidicolin, and dideoxynucleotide triphosphates (ddNTPs). DNA polymerase assays were performed as in Fig. 1A with the following modifications: lane 1, no modification; lane 2, aphidicolin (50 μg/ml) present during assay; lanes 3 and 4, preincubation in 1 mM NEM for 1 or 10 min, respectively, before assay; lanes 5 and 6, preincubation in 10 mM NEM for 1 or 10 min, respectively, before assay; lanes 7, 8, and 9, ddTTP:dTTP present during assay in molar ratios of 1:1, 5:1, and 10:1, respectively. (**B**) Trf4 DNA polymerase can use a complex DNA primer/template as substrate. A template oligonucleotide of 75 nt was hybridized to a complementary 35 mer (pair 1, lane 1), 36 mer (pair 2, lane 2), 37 mer (pair 3, lane 3), or 38 mer (pair 4, lane 4) labeled with ^{32}P at its 5′ end with T4 polynucleotide kinase in vitro. Extension of the primer/template substrate (present at 10 nM) was monitored by electrophoresis in an 8% polyacrylamide gel containing 8 M urea, after addition of Trf4 (15 nM) for 5 min at 37°C under conditions described in Fig. 1A, except that reactions contained 10 mM each of the four deoxynucleotide triphosphates (dNTPs): dTTP, dATP, dGTP, and dCTP. (**C**) Trf4 DNA polymerase activity is template directed. Reaction conditions are as described in (A), except that only one of the four dNTPs was added to each individual reaction. Each reaction contains primer/template present at 10 nM. In panel 1, the substrate used was the primer/template described in (A) (35 mer/75 mer hybrid or pair 1). Panel 2 contains the reaction products derived from a 36 mer/75 mer primer/template (pair 2) in which the primer oligonucleotide is exactly 1 nt longer than the 35 mer used in panel 1. Panel 3 products are derived from a 37 mer/75 mer (pair 3) and panel 4 from a 38 mer/75 mer hybrid (pair 4).

Table 1. Quantitation of cohesion defect in the *trf4* mutant. Cultures were grown to early logarithmic phase in YPD medium; half of the culture was arrested with α-factor and the other half with nocodazole. After 4 hours, cells were fixed and processed for FISH (*28*). Nuclei were hybridized with either a chromosome XVI *CEN*-proximal probe (11 kb from *CEN16*) or a *CEN*-distal probe (225 kb from *CEN16*) as described (*2*). The number of FISH signals was counted for each nucleus and expressed as the percentage of nuclei containing two signals for each condition. A total of 50 to 100 nuclei were counted for each probe from two independent experiments.

Strain	Genotype	% nuclei with two FISH signals			
		CEN distal		*CEN* proximal	
		Nocodazole	α-factor	Nocodazole	α-factor
CY143	Wild type	13	10	12	12
CY882	*trf4Δ::TRP1*	41	11	44	14

thesis about 60 min after release and complete replication roughly 100 min after release, as expected. In contrast, the *trf4-ts trf5* double-mutant cells show a marked delay in the G_1/S transition with little evidence of DNA synthesis onset for 120 min (Fig. 3C). A similar G_1/S transition delay has been observed in the *mcd1-1* mutant (*20*), and we also observed that the *trf4 mcd1-1* double mutant is completely inviable at any temperature (*7*). This G_1/S delay is likely to represent a checkpoint-induced phenomenon because bud emergence is also delayed in both cases. Three hours after release, the majority of *trf4-ts trf5* mutant cells contain between G_1 and G_2 DNA content (Fig. 3C), and viability has dropped 10-fold (Fig. 3D). Thus, cells lacking *TRF* function do not completely replicate the genome.

Evidence that other cohesins are involved in establishment of cohesion is based primarily on the observation of S phase–specific lethality (*15*, *16*). During the single cell cycle described above, the *trf4-ts trf5* mutant cells die rapidly and are unable to complete S phase (Fig. 3D). This is likely to reflect a requirement for *TRF* function in the establishment of sister chromatid cohesion. However, if chromatids are not fully duplicated, they are naturally held together by unreplicated regions, and, furthermore, as soon as a cell completes replication it is not possible to distinguish between a role in establishment and maintenance of cohesion. Thus, we envision that replication in the absence of only *TRF4* is able to proceed because of *TRF5* function but that this enfeebled replication fork fails to properly establish cohesion.

To determine whether DNA polymerase activity per se of Trf4 is required for proper cohesion, we made a site-directed mutation in the putative active site motif, DXD, that is known from the crystal structure of rat DNA polymerase β to coordinate Mg^{2+} and to be important for catalysis (*13*). The *trf4-236* allele replaces both aspartate residues (Trf4 amino acids 236 and 238) with alanine. DNA polymerase assays of the recombinant Trf4-236 performed side by side with Trf4 show that the mutant protein's activity is greatly diminished in the oligo(dT)/poly(dA) assay (Fig. 4A). To examine the role of Trf4 polymerase activity in cohesion, we introduced the *trf4-236* mutation into the GFP-marked chromosome strain. After nocodazole arrest, the mutant shows a clear cohesion defect compared with an otherwise isogenic *TRF4*$^+$ control (Fig. 4B). Residual polymerase activity present in Trf4-236 or a partial separation of the polymerase and cohesion functions may explain why the magnitude of the defect is not as great as that observed in the *trf4*Δ strain.

The question of whether Trf4 DNA polymerase activity is its overlapping essential function with *TRF5* was then addressed as follows. The *trf4-236* allele was integrated to replace the wild-type *TRF4* at the natural locus in strains containing either a wild-type or mutant *TRF5* allele, but expressing *TRF5* from a plasmid to ensure viability (*7*). The Trf4-236 protein was found to be expressed at a wild-type level by immunoblotting (*7*). The ability of the *trf4-236* allele to maintain viability in the absence of *TRF5* was then monitored by counterselection of the *TRF5* plasmid with 5-fluoro-orotic acid (5-FOA). The *trf4-236* allele is inviable in the absence of *TRF5* expression because it is unable to form colonies on 5-FOA medium (Fig. 4C). Thus, the Trf4 DNA polymerase activity is also required for the overlapping essential function of *TRF4* and *TRF5*.

The common *TRF4/5* function represents

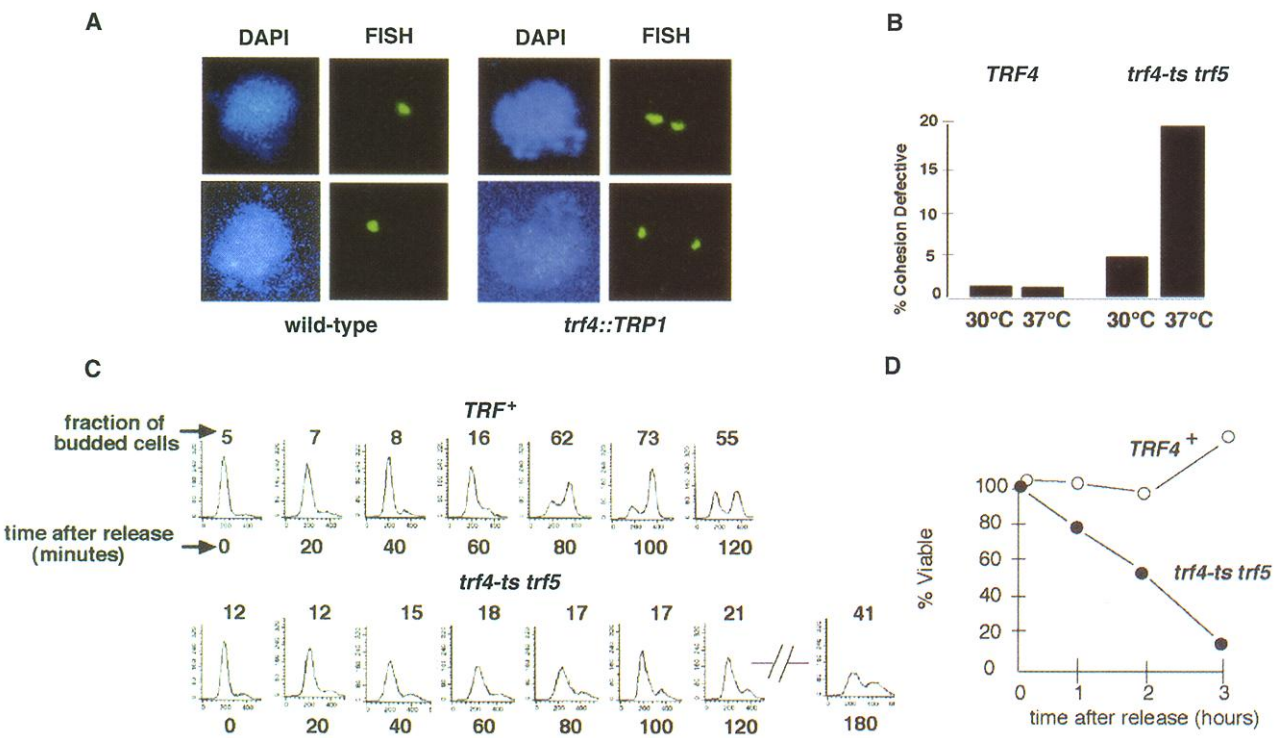

Fig. 3. (**A**) A *trf4* mutant is defective for sister chromatid cohesion. Wild-type and *trf4* mutant cells were arrested in mitosis with nocodazole, and two representative nuclei are shown after hybridization with a fluorescently labeled centromeric cosmid probe from chromosome XVI. The blue signal in the "DAPI" panel shows nuclear DNA, and the green signal in the "FISH" panel shows the positions of sister chromatids from chromosome XVI in wild-type and mutant nuclei. (**B**) *TRF4/TRF5* function is required for maintenance of cohesion. Wild-type and *trf4-ts trf5* double-mutant cells containing GFP-marked chromosome III were arrested in mitosis with nocodazole at 30°C and then shifted to 37°C for 3 hours. Cells were then examined by fluorescence microscopy for the status of sister chromatid cohesion. (**C**) A *trf4-ts trf5* double mutant is unable to complete DNA replication. Wild-type and *trf4-ts trf5* double-mutant cells were subjected to centrifugal elutriation to obtain a homogenous population of G_1 daughter cells. Cells were released into YPD medium prewarmed to 37°C, and samples were withdrawn every 20 min to examine DNA content, budding index, and viability. (**D**) A *trf4-ts trf5* double mutant dies during an abortive S phase. Viability of wild-type and *trf4-ts trf5* double-mutant cells was monitored each hour after G_1 daughter cells were shifted to 37°C by dilution and plating at the permissive temperature on YPD medium.

only the fourth essential nuclear DNA polymerase in yeast and probably in all eukaryotes. The previously observed lethality in *trf4 top1* double mutants (*1*) is likely a result of the combined loss of both the primary DNA replication swivel (*TOP1*) and another key replication factor (*TRF4*). Assays of purified human pol κ [*hTRF4-1* gene product (*4*)] also show it to be a DNA polymerase (*23*). We propose calling the yeast and human Trf4 proteins DNA polymerase κ. Recently, an *S. pombe TRF4*/pol κ family member, cid1, was found to show genetic interactions with DNA polymerase δ and ε and involvement in an S phase checkpoint (*24*). Therefore, the *TRF4*/pol κ gene family may be involved in multiple aspects of chromosome metabolism.

Genetic studies in yeast have implicated the DNA polymerase processivity factor PCNA in the establishment of cohesion (*16*), and studies of meiotic DNA synthesis and cohesion also suggest a link between the two processes (*25*). Thus, it will be important to determine whether pol κ activity is affected by PCNA. The *S. pombe* eso1 gene, which is required to establish sister chromatid cohesion, consists of two domains, one that is related to the *S. cerevisiae* cohesion establishment gene *CTF7/ECO1* and a second related to DNA polymerase η (*26*). An attractive model is that cohesion establishment factors require physical proximity to a DNA polymerase in order to execute their function. It has been suggested that repair polymerases such as pol η may travel with the replication fork and allow bypass of damaged regions that would otherwise cause fork collapse or create a terminal pause site (*12*). It may be that the establishment of cohesion is analogous in the sense that encounter of the replication fork with a cohesion site results in a switch in the DNA polymerase used to polymerize these regions. Although there have been major insights into the cohesion process recently (*27*), many questions remain regarding precisely how replication fork passage results in the establishment of effective cohesion. Our results indicate that a DNA polymerase, pol κ, provides an important link between replication and cohesion and should facilitate further elucidation of the mechanisms involved.

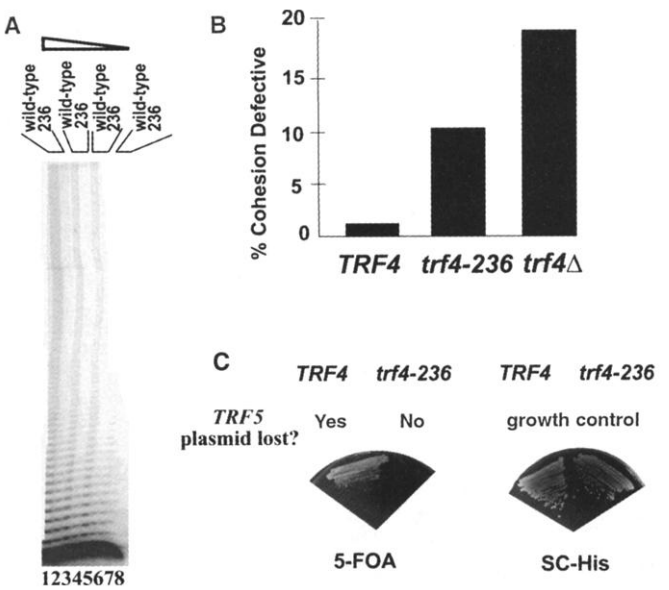

Fig. 4. (A) Trf4-236 has reduced DNA polymerase activity. Equal amounts of Trf4 or Trf4-236 were assayed for DNA polymerase activity as in Fig. 1A. Lanes 1 and 2 contain 10 nM Trf4 and Trf4-236, respectively, lanes 3 and 4 contain 5 nM, lanes 5 and 6 contain 2.5 nM, and lanes 7 and 8 contain 1.25 nM. The Trf4 and Trf4-236 proteins were purified identically. **(B)** *trf4-236* is partially cohesion defective. Cohesion assays in wild-type and *trf4-236* mutant cells with the GFP-marked chromosome (*21*, *29*). **(C)** *trf4-236* is inviable in the absence of *TRF5*. A *trf4-236 trf5/pTRF5.URA3* strain (CY1262) or an otherwise isogenic *TRF4*$^+$ parent strain (CY1231) was grown nonselectively for the *TRF5.URA3* plasmid to single colonies on YPD medium and examined for the presence of spontaneous segregants that had lost the plasmid with 5-FOA counterselection. The *TRF4*$^+$ strain is able to remain viable in the absence of *TRF5* as segregants can grow on 5-FOA medium (left panel), whereas the *trf4-236* strain cannot survive in the absence of *TRF5* because it is unable to grow on 5-FOA medium.

Table 2. Quantitation of cohesion defect in the *trf4* mutant. Strains containing the *lacI-GFP* fusion and 256 tandem repeats of the *lacO* integrated at the *LEU2* locus on chromosome III were grown in SC-his to induce the Lac-GFP repressor; cells were pelleted, resuspended in YPD with nocodazole, and incubated for an additional 4 hours. Cells were fixed and adhered to glass slides. The number of fluorescent signals (*GFP*) was counted for each nucleus and cell. At least 80 nuclei were counted for each strain from two independent experiments.

Strain	Genotype	% cells with two GFP signals	
		Nocodazole	α-factor
CY1244	Wild type *GFP-lacI* $(lacO)_{256}$	2.5	1.5
CY1247	*trf4Δ::TRP1 GFP-lacI* $(lacO)_{256}$	18	<2
	3 hours at 37°C		
CY1356	*TRF4*$^+$ *cdc16-ts GFP-lacI* $(lacO)_{256}$	2	
CY1358	*trf4 cdc16-ts GFP-lacI* $(lacO)_{256}$	15	

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6. Trf4-6HIS was purified from 6 liters of *E. coli* culture after a 3-hour induction from the T7 promoter in strain BL21DE3 codon$^+$ RIL (Stratagene). Cell extracts were made in 20 mM tris (pH 8.0), 500 mM NaCl, 10% glycerol, 1 mM dithiothreitol (DTT), and 1 mM EDTA in the presence of multiple protease inhibitors: aprotinin (1 μg/ml), leupeptin (1 μg/ml), pepstatin (1 μg/ml), chymostatin (1 μg/ml), and 1 mM phenylmethylsulfonyl fluoride. A 0.3% polyethylenimine precipitation was performed on the extract, and the supernatant was then fractionated with 0 to 40% ammonium sulfate. The pellet was resuspended in 20 mM tris (pH 8.0), 500 mM NaCl, 20 mM imidazole, 10% glycerol, 1 mM 2-mercaptoethanol, and protease inhibitors as above (starting buffer) and applied to a 1-ml Ni^{2+} agarose affinity column. The column was washed with 20 ml of 20 mM imidazole in starting buffer and 20 ml of 50 mM imidazole in starting buffer and eluted with a gradient of 50 to 500 mM imidazole in starting buffer. Fractions containing Trf4-6HIS were pooled and applied to a 1-ml mono Q anion exchange column in 250 mM NaCl, 20 mM tris (pH 8.0), 10% glycerol, 1 mM DTT, and 0.05% Tween-20. The column was washed and then eluted with a gradient of 0.25 to 1.25 M NaCl in the same buffer. Fractions containing Trf4-6HIS were pooled (500 to 600 mM salt), and glycerol was added to a final concentration of 50%. Samples were stored at −20°C in aliquots. The Trf4Δ240 was also histidine-tagged and purified identically, except that the ammonium sulfate precipitation was omitted.
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28. Fluorescence in situ hybridization assays of sister chromatid cohesion: Yeast strains were grown in YPD and arrested with either α-factor (25 μg/ml) or nocodazole (20 μg/ml). Fixation and hybridization were carried out essentially as described (*19*) with the modifications described (*2*). Slides were mounted with antifade solution containing DAPI (1 mg/ml r-phenylenediamine, 1 μM DAPI, 1× phosphate-buffered saline in 90% glycerol) and viewed with a Nikon epifluorescence microscope. Images were captured digitally with a Princeton Instruments charge-coupled device camera and IP Lab Spectrum software.
29. Cells containing the *GFP-lacI* fusion were grown overnight in SC-histidine at 30°C. Cells were diluted to an absorbance at 600 nm (A_{600}) of 0.2 and allowed to double once more. Cells were spun and resuspended in the same volume of YPD plus nocodazole (20 μg/ml) or α-factor (25 μg/ml). After 4 hours, cells were fixed by adding 0.1 volumes of 37% formaldehyde, incubated for 5 min, and processed as described (*21*). Slides were viewed with a Nikon epifluorescence microscope with a 100× oil immersion lens and a *GFP* LP filter from Chroma Technology (Brattleboro, VT).
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Cell-Cell Signaling and Movement by the Floral Transcription Factors LEAFY and APETALA1

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LEAFY (*LFY*) and *APETALA1* (*AP1*) encode unrelated transcription factors that activate overlapping sets of homeotic genes in *Arabidopsis* flowers. Sector analysis and targeted expression in transgenic plants were used to study whether *LFY* and *AP1* can participate in cell-cell signaling between and within different layers of the floral meristem. *LFY* signaled equally well from all layers and had substantial long-range action within layers. Nonautonomous action of *LFY* was accompanied by movement of the protein to adjacent cells, where it directly activated homeotic target genes. In contrast, *AP1* had only limited nonautonomous effects, apparently mediated by downstream genes because activation of early target genes by *AP1* was cell-autonomous.

Shoots and flowers are derived from collections of stem cells called meristems, which are stratified into distinct cell layers. In many plants, restrictions in the plane of cell division in the two outer layers lead to the generation of three cell lineages—the epidermal layer (L1), the subepidermal layer (L2), and the internal layer (L3)—thus allowing the generation of genetically mosaic shoots and flowers. Mosaic studies have shown that some floral transcription factors can signal from layer to layer, although signaling within layers always appeared to be largely absent (*1–6*).

Although cell-cell communication initiated by transcription factors is not unusual, plant cells differ from animal cells in that they are connected by plasma membrane–lined channels called plasmodesmata, which provide cytoplasmic continuity between adjacent cells. On the basis of the precedence of intercellular trafficking of viral proteins, it has been proposed that cell-cell communication by trafficking of transcription factors is a widespread phenomenon in plants (*7*). Two transcription factors, KN1 in maize and DEF in *Antirrhinum*, have indeed been shown to move to cells in which their RNAs are not found (*4, 8, 9*). However, because direct target genes have not been conclusively identified for either factor, the biological activity of the exported proteins could not be assayed, although DEF movement at late stages of development correlated with some nonautonomous phenotypic effects during early stages (*4*).

To investigate transcription factor trafficking in *Arabidopsis* flowers, we used two complementary approaches to compare the cellular autonomy of LFY and AP1, two unrelated transcription factors that activate overlapping sets of target genes (*10, 11*). In the first approach, we used FLP recombinase to create genetically mosaic plants with sectors marked by excision of a β-glucuronidase (GUS) gene. Activation of *FLP* under the control of a heat shock promoter (*HSP::FLP*) (*12*) resulted in *35S::AP1*$^+$ *GUS*$^-$ sectors in a *35S::AP1*$^-$ *GUS*$^+$ *ap1-1* background (*13*). In *ap1-1* flowers, first-whorl sepals are replaced by bracts in the axils of which secondary flowers arise, whereas second-whorl petals are typically absent (*14*). Analysis of mosaic shoots from heat-shocked *ap1-1 HSP::FLP FLP.AP1* plants revealed that the recombined allele had to be present in all layers for full rescue and that clones expressing *35S::AP1* only in L3 were indistinguishable from *ap1-1* mutants (*13*).

Clones expressing *35S::AP1* only in L1 produced first-whorl organs with L1 cells typical of wild-type sepals, but L2 and L3 cells more typical of *ap1* bracts (Fig. 1D). Second-whorl organs were restored, and these had petal identity in L1 but not in the internal layers (Fig. 1E). Conversely, expression of *35S::AP1* in L2 and L3 produced first-whorl organs with sepal anatomy in the internal layers, but a bract-like L1 (Fig. 1F). In the second whorl, organs with petal shape were produced, but L1 typically lacked petal identity (*13*). None of the L1, L2, or L3 clones suppressed the formation of secondary flowers (*13*). Mericlinal sectors, in which *35S::AP1*$^+$ and *35S::AP1*$^-$ cells abutted in the same layer, showed complete autonomy of *AP1* within layers (Fig. 1F). In summary, these genetic mosaics revealed that *AP1* acts largely cell-autonomously to control cellular identity, but nonautonomously to promote outgrowth of second-whorl organs.

A strategy similar to that for *AP1* was used to generate *35S::LFY* sectors in a *lfy-12* mutant background (*13*). Mosaic plants were obvious because they produced flowers with

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