Mitotic chromosome condensation in the rDNA requires *TRF4* and DNA topoisomerase I in *Saccharomyces cerevisiae*

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DNA topoisomerase I (topo I) is known to participate in the process of DNA replication, but is not essential in *Saccharomyces cerevisiae*. The *TRF4* gene is also nonessential and was identified in a screen for mutations that are inviable in combination with a *top1* null mutation. Here we report the surprising finding that a *top1 trf4-ts* double mutant is defective in the mitotic events of chromosome condensation, spindle elongation, and nuclear segregation, but not in DNA replication. Direct examination of rDNA-containing mitotic chromosomes demonstrates that a *top1 trf4-ts* mutant fails both to establish and to maintain chromosome condensation in the rDNA at mitosis. We show that the Trf4p associates physically with both Smc1p and Smc2p, the *S. cerevisiae* homologs of *Xenopus* proteins that are required for mitotic chromosome condensation in vitro. The defect in the *top1 trf4-ts* mutant is sensed by the *MAD1*-dependent spindle assembly checkpoint but not by the *RAD9*-dependent DNA damage checkpoint, further supporting the notion that chromosome structure influences spindle assembly. These data indicate that *TOP1* (encoding topo I) and *TRF4* participate in overlapping or dependent steps in mitotic chromosome condensation and serve to define a previously unrecognized biological function of topo I.

[Key Words: DNA topoisomerase; chromosome condensation; mitosis; nuclear division; checkpoint]

Received July 29, 1996; revised version accepted August 26, 1996.

Chromosomes are involved in dynamic cellular processes such as DNA replication, transcription, chromatin assembly, mitotic condensation, and genetic recombination that can lead to the formation of local domains of torsional stress [for review, see Wang and Lynch 1993]. In *Saccharomyces cerevisiae*, DNA topoisomerases I and II (topo I and II) appear to act together during DNA replication as a swivel to relieve torsional stress at the replication fork. When both topo I and II are inactivated, DNA replication stops rapidly [Brill et al. 1987], with elongation of new DNA chains continuing for only a few thousand nucleotides [Kim and Wang 1989]. Transcription can also lead to the formation of locally supercoiled domains in DNA. In the twin domain model [Liu and Wang 1987], movement of a transcription complex along the helical backbone generates positive supercoils ahead of the complex and negative supercoils behind the complex. In *S. cerevisiae*, topo I and II function together to facilitate rRNA transcription and, to a lesser extent, mRNA transcription [Brill et al. 1987].

Despite the considerable evidence for the involvement

of topo I in both DNA replication and transcription, topo I is not essential in either *S. cerevisiae* or *Schizosaccharomyces pombe*. Null mutations in *TOP1*, the gene encoding the only type I DNA topoisomerase activity detectable in crude extracts, cause only modest growth defects [Thrash et al. 1984; Uemura and Yanagida 1984; Thrash et al. 1985]. Furthermore, the overall rates of both DNA and RNA synthesis are normal in *top1* mutants [Brill et al. 1987], supporting the suggestion that topo II activity can substitute for topo I activity in these crucial processes [Uemura and Yanagida 1984; Goto and Wang 1985; Brill et al. 1987]. In both *S. cerevisiae* and *S. pombe* conditional *top2* mutations exacerbate the growth defect of *top1* cells [Goto and Wang 1985; Uemura et al. 1987a]. This demonstrates that some of the functions of type I and type II topoisomerases overlap. It remains unclear, however, whether topo I has additional functions distinct from those of topo II.

To investigate further the in vivo functions of topo I, we performed a genetic screen to identify mutations affecting gene products that perform overlapping or dependent functions with topo I and, thereby, to elucidate further which processes in the cell require topo I [Sadoff et al. 1995]. We have identified four complementation groups of mutants with this phenotype called *TRF*, for

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DNA Topoisomerase I-Related Function (Sadoff et al. 1995; Zhu et al. 1995; Castaño et al. 1996). The predicted Trf4 protein shares homology with the amino-terminus of Top1p (Sadoff et al. 1995) and is a member of a novel gene family that is evolutionarily conserved (Castaño et al. 1996). Null mutations in *trf4* display only a slight growth defect but are completely inviable in combination with a *top1* null mutation despite having a normal level of active topo II (Sadoff et al. 1995). Thus, we have investigated the reason for the inviability of the *top1 trf4* double mutant.

Whereas topo II is known to be required for mitotic chromosome condensation both in vitro (Adachi et al. 1991; Hirano and Mitchison 1993) and in vivo (Uemura et al. 1987b) and for separating intertwined, replicated chromosomes at the time of mitosis (DiNardo et al. 1984; Holm et al. 1985), there was little evidence to suggest the involvement of a type I DNA topoisomerase in mitotic events. An evolutionarily conserved class of proteins required for mitotic chromosome condensation have been identified using a combination of a *Xenopus* cell-free system (Hirano and Mitchison 1994) and fungal genetics (Saka et al. 1994; Strunnikov et al. 1993, 1995). The *Xenopus* XCAP-C and XCAP-E proteins are part of a protein complex required for condensation in vitro (Hirano and Mitchison 1994) and are related to *S. cerevisiae* Smc1 and Smc2 proteins, respectively (Strunnikov et al. 1993, 1995). The Smc1p and Smc2p proteins are also associated with each other in vivo (Strunnikov et al. 1995). Whereas it had been thought that *S. cerevisiae* chromosomes may be too small to necessitate significant condensation at mitosis to facilitate segregation, recent studies using fluorescently labeled probes demonstrate that condensation does occur in yeast (Guacci et al. 1994; Saka et al. 1994) and that the genes required for condensation in yeast are related to those required for condensation in vertebrates (Hirano and Mitchison 1994; Hirano 1995).

We report here the unexpected result that a *top1 trf4-ts* double mutant is defective in the mitotic events of chromosome condensation, spindle elongation and nuclear segregation, but not in DNA replication at the restrictive temperature. Furthermore, we show that genetic and physical interactions are observed between the *TRF4* and the *SMC* genes and proteins, which are the *S. cerevisiae* homologs of *Xenopus* proteins required for chromosome condensation in vitro. These data indicate that *TOP1* and *TRF4* participate in overlapping or dependent steps in mitotic chromosome condensation and serve to define a previously unrecognized biological function of topo I.

Results

A top1 trf4-ts double mutant dies at the time of mitosis

A *TRF4* allele was identified in a screen for mutants with *TOP1* (encoding topo I)-requiring mutations. To determine the cause of the inviability of the *top1 trf4* double mutant, we isolated an allele of *trf4* that was tempera-

ture sensitive for growth in a *top1* genetic background and replaced the wild-type *TRF4* allele with the *ts* allele to generate a *top1 trf4-ts* double mutant (see Materials and Methods). The *trf4-ts* allele is recessive for inviability in combination with *top1* and, therefore, is likely to be a loss-of-function mutation. To determine whether the common function performed by *TOP1-TRF4* is required at a specific time in the cell cycle, we synchronized cultures of the *top1 trf4-ts* mutant in the G₁ phase of the cell cycle using α -factor at a permissive temperature (24°C), and released cells from the block in liquid culture by α -factor withdrawal at either 24°C or at a restrictive temperature (37°C) while monitoring both cell number and cell viability following release. The 24°C culture displayed the expected constant cell number and number of colony-forming units (cfu) for 1.5 hr, followed by an approximately twofold increase in cell number and in the number of colony forming units, resulting from cell division, between 1.5 and 2.5 hr after release (Fig. 1A). The 37°C culture also maintained the original cell number and number of cfu for 1.5 hr after release. However, whereas cell division occurred at 37°C between 1.5 and 2.5 hr, just as it had at 24°C, the number of cfu (viable cells) failed to increase at all upon cell division and subsequently began to decrease (Fig. 1A).

When the *top1 trf4-ts* mutant was shifted to the restrictive temperature without the removal of α -factor so that cells remained in G₁, very little loss of viability was observed. Figure 1B shows the ratio of cfu (viable cells) to cell number on the y-axis for cultures of the *top1 trf4-ts* mutant that were synchronized in G₁ with α -factor as described above and then either released from the block at 24°C or 37°C or held at 37°C in the presence of α -factor. The 37°C culture that remains in G₁ because of the continued presence of α -factor shows approximately fourfold greater viability (cfu/cell) than the culture that was allowed to proceed through mitosis. These data indicate that the *top1 trf4-ts* mutant grown at the restrictive temperature undergoes a cell division that results in inviable progeny in the first cell cycle following temperature shift and, furthermore, that *top1 trf4-ts* cells maintained in G₁ for the same time period at the restrictive temperature largely remain viable.

Spindle elongation and nuclear division are defective in the top1 trf4-ts mutant

Kinetic analysis of DNA content by flow cytometry following α -factor release shows that wild-type and *top1 trf4-ts* cultures complete DNA synthesis at the same rate at the restrictive temperature of 37°C (Fig. 2A). In addition, the rate of DNA synthesis is the same at both 24°C and 37°C for the *top1 trf4-ts* culture (not shown). This indicates that there is no gross failure to complete DNA synthesis in the *top1 trf4-ts* mutant at the restrictive temperature. Whereas DNA synthesis is completed at a wild-type rate in the *top1 trf4-ts* mutant, 4'6-diamidino-2-phenylindole (DAPI) staining revealed several defects in nuclear division by 1.5 hr after α -factor release. The 37°C culture displayed many cells in which the nu-

Figure 1. A *top1 trf4-ts* mutant dies at the time of mitosis. (A) Cells of strain CY950 (*MATa top1 trf4-ts*) were grown to logarithmic phase in YPD at 24°C and synchronized in the G₁ phase with α -factor. After 1.5 generations cells were harvested, washed, and resuspended in fresh medium. One half of the culture was further incubated at 24°C and the other half at 37°C. Samples were withdrawn every 30 min, diluted in sterile water and appropriate dilutions were plated on YPD plates at 24°C to determine the number of colony forming units (cfu). (B) Cells were treated as above except that α -factor was not removed from one culture at 37°C. The number of cells was determined by counting each sample in a hemocytometer under a light microscope.

cleus appears unevenly divided between the mother and daughter cells upon spindle elongation, indicative of aberrant nuclear division (see arrows in Fig. 2B). Cells with unevenly divided nuclei constitute 37% of those undergoing nuclear division at the restrictive temperature (Table 1). Unevenly divided nuclei were not observed at the permissive temperature. In addition, after 1.5 hr at 37°C, 36% of *top1 trf4-ts* cells appeared to have failed to position the nucleus in the bud neck, whereas none of the cells at 24°C failed to (Table 1). Anucleate (10% at 2 hr) and binucleate cells (7% at 2 hr) were also observed at the restrictive but not permissive temperatures. Consistent with the results obtained using synchronized cultures, the terminal-arrest morphology of asynchronous cultures of the *top1 trf4-ts* mutant at 37°C included 28% large-budded cells, 10% small-budded cells and 62% unbudded cells ($n = 113$), indicating that a uniform cell-cycle arrest does not result from the loss of *TOP1-TRF4* function, but that many cells proceed through a defective mitosis.

Figure 2. DNA content, nuclear and spindle morphology in the *top1 trf4-ts* mutant. (A) DNA content of the *top1 trf4-ts* mutant and an isogenic wild-type parent strain at 0.5 hr intervals following synchronization with α -factor at 24°C and release at 37°C. (B) Nuclear and spindle morphology of cells 1.5 hr after release from the α -factor block at 24°C or 37°C. Cells were fixed and stained with DAPI to visualize DNA and with antitubulin antibody to reveal microtubules. Small arrows denote cells showing unevenly or incompletely divided nuclei that have anaphase spindles. Neither *top1* nor *trf4* single mutants or the wild-type parent strain show aberrant spindles or nuclear morphology at 37°C.

The synchronous cultures described above were also examined for spindle morphology 1.5 hr after release from the α -factor block. Examination of the mitotic spindle by indirect immunofluorescence using an antitubulin antibody shows that the 24°C culture consists primarily of cells with long, anaphase spindles, whereas the 37°C culture contains few cells with anaphase spindles and primarily cells with short bipolar spindles (Fig. 2B and Table 2). Examination of the 37°C culture up to 3 hr after release showed that few anaphase spindles are ever established (Table 2). The anaphase spindles that were observed at 37°C appeared much thinner and stained more faintly than normal spindles. Neither *top1* nor *trf4* single mutants, nor our wild-type parent strain showed spindle defects at 37°C. Thus, *top1 trf4-ts* cells are able to complete DNA synthesis and establish a short bipolar

Table 1. Nuclear morphology and position in *top1 trf4-ts*

Time after release	Small- and medium-budded		Large-budded (with two distinct nuclear masses) (%)	
	nucleus in budneck (%)	nucleus not in budneck (%)		
24°C				
1 hr	70 (0%) ^a	15	5	<i>n</i> = 59
1.5 hr	23 (0%) ^a	0	48	<i>n</i> = 63
37°C				
1 hr	34 (20%) ^a	32	0	<i>n</i> = 105
1.5 hr	39 (37%) ^a	36	<1	<i>n</i> = 123

Cells from the experiment shown in Fig. 2 were counted under a fluorescence microscope (*n*, number of cells counted).

^aPercent that contained a nucleus that was unevenly distributed between mother and daughter cells.

spindle at the restrictive temperature, but subsequently fail to elongate the mitotic spindle properly and complete nuclear division.

trf4 and *top1* mutants are defective in centromere-mediated plasmid segregation

Plasmid loss assays have been used to distinguish between defects in DNA replication and segregation (Strunnikov et al. 1993). Mutants defective in plasmid replication are expected to display increased loss rates for both centromere and 2 μ plasmids, whereas mutants with a centromere-mediated segregation defect are expected to display a high loss rate of *CEN* plasmids only. The mitotic stability of two different plasmids marked with *URA3* and either *CEN* or 2 μ sequences was measured by determining the percentage of cells in a culture that retained the plasmid under selection (Strunnikov et al. 1993). A *trf4* single deletion mutant displayed a 10-fold higher rate of centromere plasmid loss than an oth-

Table 2. Spindle morphology in *top1 trf4-ts*

Time (h)	Long anaphase spindles	Short bipolar spindles	G ₁ -like asters	
24°C				
0	0	0	100	(<i>n</i> = 133)
0.5	0	8	92	(<i>n</i> = 135)
1	13	43	43	(<i>n</i> = 106)
1.5	62	23	15	(<i>n</i> = 127)
2	6	13	82	(<i>n</i> = 120)
3	22	48	30	(<i>n</i> = 28)
37°C				
0	0	0	100	(<i>n</i> = 133)
0.5	0	1	99	(<i>n</i> = 117)
1	5	61	33	(<i>n</i> = 107)
1.5	3	80	17	(<i>n</i> = 115)
2	6	46	47	(<i>n</i> = 79)
3	7	40	47	(<i>n</i> = 15)

T = 0 is defined as the time immediately after α -factor release; *n*, number of cells counted.

Figure 3. *trf4* and *top1* null mutants display an increased rate of *CEN* plasmid loss. Cultures of wild-type (CY184), $\Delta top1-7::LEU2$ (CY185) and $\Delta trf4-101::HIS3$ (CY855) strains were transformed with either pYEp24 (2 μ) (hatched bars) or pRS316 (*CEN*) (gray bars) plasmids to Ura⁺. Cells were grown to exponential phase (A_{600} = 1.5) in SC-Ura medium at (A) 30°C or (B) at 24°C, and aliquots were then diluted in sterile water and plated on medium containing uracil. Replica prints were made to SC-Ura plates to calculate the percentage of colonies that had lost the plasmid.

erwise isogenic wild-type strain at 30°C (Fig. 3A). However, the mutant did not show an increased loss rate of a 2 μ plasmid, indicating that *trf4* single mutants are defective in centromere-mediated plasmid segregation and not in plasmid replication. A *top1* single mutant did not show an increased loss rate of either centromere or 2 μ plasmids at 30°C, but at 24°C a threefold increased rate of *CEN* plasmid loss is observed, whereas no change in loss rate is observed in an isogenic wild-type strain at 24°C (Fig. 3B). This suggests that *top1* and *trf4* mutants are not defective in plasmid DNA replication but are defective in centromere-mediated plasmid segregation.

Genetic and physical interaction between TRF4 and SMCs

Several of the phenotypes of the *top1 trf4-ts* mutant, such as failure to elongate the mitotic spindle, the presence of unevenly divided nuclei and an increased rate of centromere plasmid loss, are shared by mutants defective in *SMC1* (Strunnikov et al. 1993) or *SMC2* (Strunnikov et al. 1995). The *SMC1* and *SMC2* genes are the *S. cerevisiae* homologs of *Xenopus* XCAP-C and XCAP-E, proteins required for mitotic chromosome condensation in vitro (Hirano and Mitchison 1994). To determine whether *TRF4* might also function in chromosome condensation, we attempted to make double mutants by crossing a strain containing a *trf4* null mutation (CY855) and a strain carrying an *smc1-2* point mutation (3aAS273; see Materials and Methods). In 46 tetrads dissected from this cross, we recovered 43 (of the expected 46) *SMC1*⁺*TRF4*⁺ meiotic segregants, 24 *SMC1*⁺*trf4*⁻ segregants, 33 *smc1*⁻*TRF4*⁺ segregants but no *smc1*⁻*trf4*⁻ segregants after 3 days at 24°C (Table 3). However, after 6 days at 24°C seven apparent *smc1-2 trf4*⁻ segregants were recovered as pinpoint colonies. This indicates that *smc1-2 trf4* double mutants have a severe

Table 3. Genetic interaction between *smc1-2* and *trf4*

Genotype recovered	Number of spores after 3 days	Number of pinpoint colonies after 6 days	Total expected
<i>SMC1 TRF4</i>	43	0	46
<i>smc1-2::LEU2 TRF4</i>	33	0	46
<i>SMC1 trf4-101::HIS3</i>	24	0	46
<i>smc1-2::LEU2 trf4-101::HIS3</i>	0	7	46

Strain CY890 (*MATa Δtrf4-101::HIS3*) was crossed with strain 3aAS273 (*MATa smc1-2::LEU2*) at 24°C and sporulated. Tetrads were dissected on YPD plates and incubated at 24°C for 6 days. After colonies had grown, the plates were replica-printed to SC-his or SC-leu plates to identify single and double mutants. All of the double mutants formed were very tiny colonies (pinpoint colonies) at 24°C.

growth defect relative to either single mutant. In contrast, *trf4 smc2-6* double mutants were readily recovered and did not show a synthetic growth defect (not shown).

The XCAP-E protein is part of a protein complex required for mitotic chromosome condensation in vitro (Hirano and Mitchison 1994). The *S. cerevisiae* homolog, Smc2p, is also required for mitotic chromosome condensation and is physically associated with Smc1p (Strunnikov et al. 1995). Therefore, we sought to determine whether Smc1p, Smc2p, and Trf4p were part of the same protein complex using coimmunoprecipitation. The Smc proteins are present in very low abundance and their interaction has only been detected upon overexpression (Strunnikov et al. 1995). Similarly, it was necessary to overexpress Trf4-PYP to detect it by Western blotting. Therefore, to test for interaction a 2-μ plasmid carrying either *SMC1* epitope tagged with the MYC epitope or *SMC2* epitope tagged with the HA epitope (Strunnikov et

al. 1995) was introduced into a *trf4* null mutant, as was a second plasmid carrying a galactose-inducible *TRF4* gene tagged with a polyoma epitope (PY; Grussenmeyer et al. 1985). Extracts were prepared from the strain grown either on glucose or galactose, and the Trf4-PY protein was immunoprecipitated with an anti-PY monoclonal antibody coupled to protein G–Sepharose beads (Harlow and Lane 1988). Immunoprecipitation of Trf4-PY using α-PY antibodies from extracts of strains grown on galactose results in coimmunoprecipitation of both Smc1–MYCp and Smc2–HAp, as revealed by Western analysis of immunoprecipitates (Fig. 4). In contrast, immunoprecipitation from extracts of the same strain grown in glucose, a condition under which Trf4–PY is not expressed, does not result in coimmunoprecipitation of Smc2–HAp or Smc1–MYCp (Fig. 4). No coimmunoprecipitation of Trf4–PY and Top1–HAp is observed under these conditions. Thus, Trf4p is physically associated with Smc1p and Smc2p, but not with Top1p in vivo.

TOP1–TRF4 function is required for the formation and maintenance of mitotic chromosome condensation in the rDNA

A genetic interaction between an *smc1-2* and a *trf4* mutation and a physical interaction between the Smc1, Smc2, and Trf4 proteins suggested that the *top1 trf4-ts* mutant might be defective in the process of mitotic chromosome condensation. Therefore, we used a fluorescence in situ hybridization (FISH) assay (Guacci et al. 1994) to examine mitotic chromosome condensation in the *top1 trf4-ts* mutant directly. In this assay, cell walls are removed by enzymatic digestion and spheroplasts are adhered to a glass slide and fixed. Biotinylated DNA probes are then hybridized in situ to the nuclei and visualized using labeled avidin to examine chromosome structure (Guacci et al. 1994). The most dramatic changes in chromosome structure during the cell cycle are seen using rDNA probes (Guacci et al. 1994), presumably because of the large size of the array. Using this assay, wild-type cells arrested in G₁ display a large amorphous fluorescent signal on the edge of the chromosomal mass that is similar to the pattern obtained after staining for nucleolar proteins. In contrast, cells arrested in G₂/M show intense discrete line-like fluorescent signals (Guacci et al. 1994). Figure 5A shows the difference in structure of rDNA at G₁ (α-factor block) versus G₂/M (1.5 hr after release from the block) in our wild-type strain. rDNA at G₁ appears as a dispersed signal on the edge of the nucleus. We have termed this nuclear appearance a puff. In contrast, at G₂/M 60% of nuclei contain rDNA that appears to be more compact and string-like (Fig. 5A). We have termed these nuclei strings. Even at G₂/M, only about 60% of nuclei appear to contain strings in our wild-type strain (Fig. 5A and Table 4). This may indicate that the fixation procedure results in a partial disruption of the condensed state or that the cultures were not synchronized perfectly. These results are consistent with the cell-cycle-dependent changes in rDNA structure reported previously (Guacci et al. 1994).

Figure 4. Coimmunoprecipitation of Trf4p, Smc1p, and Smc2p. A strain containing a *Δtrf4-101::HIS3* null allele, was cotransformed with 2 plasmids: *pGAL1::TRF4-PY* (pCB787) and a 2-μ plasmid that expresses either *SMC1-MYC* (pAS258), *SMC2-HA* (pAS428) or *TOP1-HA* (pCB119). The strains were grown in either glucose- or galactose-containing medium. Protein extracts were immunoprecipitated with anti-PY antibody coupled to protein G–Sepharose beads and the precipitated material was loaded on an SDS/PAGE gel and transferred to nitrocellulose. The blots were probed with anti-MYC antibodies to detect Smc1-MYCp or anti-HA antibodies to detect the presence of Smc2-HAp or Top1-HAp.

Figure 5. rDNA chromosome condensation in wild-type and the *top1 trf4-ts* mutant. Cultures of wild-type (CY143) and *top1 trf4-ts* (CY950) strains were synchronized with α -factor and released at either 24°C or 37°C, and samples were withdrawn 1.5 hr after release, fixed, and spheroplasted. Nuclei were adhered to glass slides and hybridized with a biotinylated rDNA probe. (A) rDNA at different stages of the cell cycle in the wild-type strain immediately after release from α -factor (G_1) or 1.5 hr after release at 37°C (G_2/M). Arrowheads at left point to the amorphous fluorescent signals characteristic of cells in the G_1 phase of the cell cycle which we term puffs; small arrows at right show the more condensed, line-like signal (strings) that appear at the time of mitosis. (B) rDNA structure 1.5 hr after release from α -factor at either 24°C or 37°C in the *top1 trf4-ts* mutant. (C) Cells were arrested with 20 μ g/ml of nocodazole at 24°C and, while arrested, half of the culture was shifted to 37°C and half was kept at 24°C for an additional 1.5 hr. Triangular arrowheads at left denote the uncondensed chromatin (puffs) and small arrows denote the condensed state (strings); arrows at right panel show partially decondensed nuclei that are observed at 37°C. Bar, 5 μ m.

Synchronous cultures of a *top1 trf4-ts* double mutant generated using α -factor block and release were exam-

Table 4. Failure to condense rDNA in the *top1 trf4-ts* mutant

Strain	Temperature	"strings" (%)	"puffs" (%)
Wild-type	24°C	65	35
Wild-type	37°C	57	42
$\Delta trf4-101::HIS3$	24°C	30	69
$\Delta trf4-101::HIS3$	37°C	33	66
$\Delta top1-7::LEU2$	24°C	38	61
$\Delta top1-7::LEU2$	37°C	31	67
$\Delta top1-7::LEU2 trf4-ts$	24°C	37	63
$\Delta top1-7::LEU2 trf4-ts$	37°C	0.5	99

Cells of wild-type (CY143), $\Delta top1-7::LEU2$ (CY920), $\Delta trf4-101::HIS3$ (CY890), and $\Delta top1-7::LEU2 trf4-ts$ (CY950) were synchronized with α -factor and released at 24°C or 37°C. Samples were taken 1.5 hr after release and examined by FISH to assay chromosome condensation as described in Materials and Methods. Slides were viewed under the microscope and counted ($n > 150$ for each strain and condition). A "puff" is a large amorphous signal that is on the edge of the nucleus characteristic of the uncondensed chromatin of cells in the G_1 phase of the cell cycle; a "string" is the more condensed, line-like fluorescent signal that is observed in the G_2/M phase of the cell cycle.

ined for rDNA structure using the FISH assay. After 1.5 hr at 24°C, 37% of the cells from the *top1 trf4-ts* culture contained rDNA in the condensed or string state and 63% contained puff nuclei (Fig. 5B), a slight (twofold) reduction in the number of condensed nuclei compared with wild-type cultures. In contrast, the 37°C *top1 trf4-ts* culture almost completely failed to form string nuclei, with >99% of nuclei remaining as puffs (Fig. 5B). Further incubation of the 37°C cultures for up to 2.5 hr after α -factor block/release resulted in no increase in the fraction of string nuclei (data not shown). Cultures of *top1* or *trf4* single mutants showed a twofold reduction in the number of nuclei with condensed rDNA compared with wild-type at either temperature (Table 4), but were clearly able to form condensed structures. Thus, the *top1 trf4-ts* double mutant is unable to condense rDNA at the time of mitosis.

To determine whether *TOP1-TRF4* function is required not only to establish but also to maintain condensed rDNA chromatin, we arrested cultures of the *top1 trf4-ts* mutant with nocodazole at 24°C, a point at which rDNA condensation has already occurred (Guacci et al. 1994), and then shifted the cells to either 24°C or 37°C in the continued presence of nocodazole. The arrested *top1 trf4-ts* culture at 24°C contained the expected 38% of nuclei with condensed rDNA and, after 1.5 hr of further incubation at 24°C in the presence of nocodazole, 95% of the condensed nuclei remained condensed (Fig. 5C and Table 5). In contrast, for cells shifted to 37°C, only 35% of the original condensed nuclei remained condensed (Fig. 5C and Table 5), indicating that the majority of condensed nuclei underwent decondensation within 1.5 hr at the restrictive temperature. In addition, what appear to be partially decondensed nuclei were observed

Table 5. Percent of “strings” and “puffs” after nocodazole arrest at 24°C in *top1 trf4-ts*

Treatment	Original “strings” remaining after 1.5 hr (%)
24°C + nocodazole	95
37°C + nocodazole	35

Strain CY950 (*top1 trf4-ts*) was grown to logarithmic phase and nocodazole was added to a final concentration of 20 µg/ml. After 3 hr of incubation, half of the culture was shifted to 37°C in the presence of nocodazole, and the other half was kept at 24°C in the presence of nocodazole. After 1.5 hr, cells were prepared for FISH and counted under the microscope to determine the percentage of “puffs” and “strings” under each condition. *n* > 150 for each condition.

at 37°C (see large arrows in Fig. 5C). This indicates that *TOP1-TRF4* function is required both to establish and to maintain rDNA condensation at mitosis, suggesting that these proteins may play a direct role in the condensation process.

The defect in the top1 trf4-ts mutant is sensed by the spindle assembly checkpoint

Recent results suggest that chromosome structure plays a more direct role than previously appreciated in formation and function of the mitotic spindle (Li and Nicklas 1995). The spindle assembly checkpoint genes, *MAD1-3* and *BUB1-3*, detect spindle malformations and signal cell-cycle arrest (Hoyt et al. 1991; Li and Murray 1991; Murray 1995). To determine whether the failure of mitotic chromosome condensation in a *top1 trf4-ts* mutant is detected by this checkpoint system, we introduced a mutation in *MAD1* into the *top1 trf4-ts* double mutant. The *top1 trf4-ts mad1* triple mutant displays roughly 100-fold reduced viability at a semipermissive temperature (29.5°C) compared with an otherwise isogenic *MAD1*⁺ strain (Fig. 6). In contrast, little or no change in viability is observed upon introduction of a *rad9* mutation, which eliminates a checkpoint for DNA damage (Weinert and Hartwell 1988) into a *top1 trf4-ts* strain. This suggests that, in *S. cerevisiae*, failure to condense

Figure 6. The defect in the *top1 trf4-ts* mutant is sensed by the spindle assembly checkpoint. Isogenic *top1 trf4-ts* (CY950), *top1 trf4-ts mad1Δ.2* (CY970) or *top1 trf4-ts rad9Δ::URA3* (CY978) strains were grown in YPD medium at 24°C. The *A*₆₀₀ was adjusted to 1.0 and serial 10-fold dilutions were made in sterile water. 5 µl of each dilution was spotted on YPD plates and incubated at either a permissive temperature (24°C) or a semipermissive temperature (29.5°C).

chromosomes at mitosis results in an improperly formed spindle, perhaps as a result of a failure to attach microtubules to the kinetochores of uncondensed chromosomes.

We also have observed that low levels of hydroxyurea (HU), a drug that inhibits DNA synthesis, actually increases viability of the *top1 trf4-ts* mutant at least 10-fold at a semipermissive temperature (29.5°C; not shown). This phenotype is similar to *mad1* mutants, which are rescued from benomyl hypersensitivity by low levels of HU (Hardwick and Murray 1995). The longer S phase that occurs in the presence of HU presumably allows more time to correctly assemble the mitotic spindle. This further suggests that DNA synthesis is not the cause of the growth defect in the *top1 trf4-ts* mutant, because a drug that inhibits DNA synthesis increases viability of the mutant.

Discussion

In addition to the previously recognized roles of topo I in DNA replication and rRNA transcription, we have found that topo I is required, in combination with *TRF4*, for proper chromosome condensation at mitosis. Synchronous cultures of a *top1 trf4-ts* mutant display aberrant spindle elongation and nuclear division and lose viability shortly after passage through mitosis. A direct examination of mitotic chromosomes using a FISH assay indicates that the *top1 trf4-ts* double mutant fails to condense rDNA-containing chromosomes at the time of mitosis. Furthermore, *TRF4* function is required to maintain the condensed state, because a *top1 trf4-ts* mutant that has already condensed rDNA at the permissive temperature undergoes significant decondensation when shifted to the restrictive temperature. It is very likely that non-rDNA chromosomes are also affected because *trf4* and *top1* single mutants display a centromere-mediated plasmid segregation defect, and the double mutant undergoes a grossly aberrant nuclear division. We and others are currently developing chromosome painting methods to examine chromosome condensation in single-copy regions. Immunoprecipitation experiments indicate that the Trf4 protein is physically associated with Smc1p and Smc2p, the *S. cerevisiae* homologs of XCAP-C and XCAP-E, respectively. XCAP-C and XCAP-E are *Xenopus* proteins required for mitotic chromosome condensation in vitro. Smc2p is also required for the maintenance of chromosome condensation in *S. cerevisiae* (Strunnikov et al. 1995). Taken together, these data indicate that *TOP1* and *TRF4* participate in overlapping or dependent steps in mitotic chromosome condensation and serve to define a previously unrecognized biological function for topo I.

The role of topo I at mitosis

The inviability of the *top1 trf4-ts* double mutant at the restrictive temperature could result from the participation of both proteins directly in mitotic chromosome condensation or from an aberrant round of DNA replication in the absence of topo I that results in the forma-

tion of chromosomes that are poor substrates for subsequent mitotic condensation promoted by Trf4p. The aberrant round of DNA synthesis in a *top1* background might result in improper nucleosome reassembly, for example, an activity that topo I can promote in *in vitro* assays (Almouzni and Mechali 1988; Hirano and Mitchison 1991). This explanation would predict that involvement of topo I in condensation *in vitro* would be observed only in a replication-coupled system and not, for example, in the replication-independent *Xenopus* *in vitro* system (Hirano and Mitchison 1994). We favor a direct role for topo I in the formation of mitotic chromosomes for the following reasons: (1) DNA replication is completed at the same rate in the *top1 trf4-ts* mutant at either the permissive or the restrictive temperatures; (2) growth in the presence of low levels of HU, a drug that inhibits DNA synthesis, actually enhances the viability of the *top1 trf4-ts* mutant at a semipermissive temperature; (3) the defect in the *top1 trf4-ts* mutant is not sensed by the *RAD9*-dependent DNA damage checkpoint, whereas, in contrast, DNA replication in a strain with a mutant histone H4 gene that cannot be acetylated on its amino-terminal lysine residues results in a *RAD9*-dependent cell-cycle arrest (Megee et al. 1995); and (4) *top1* single mutants display an increased rate of *CEN* plasmid nondisjunction at 24°C, indicating that *TOP1* is required for proper centromere-mediated segregation even in the presence of *TRF4*.

Topo I activity per se is required for the mitotic function of *TOP1* because a mutation in the active site tyrosine (Y727F) is completely unable to complement the *top1 trf4* defect (I.B. Castaño and M.F. Christman unpubl.). Thus, topo I is unlikely to be a structural component of mitotic chromosomes but rather its topoisomerase activity is required for condensation, perhaps to relieve torsional stress associated with condensation. This interpretation is consistent with the apparent lack of direct association of Top1p and Trf4p. Topo II is required for chromosome condensation in *S. pombe* (Uemura et al. 1987b) and in *Xenopus* extracts (Adachi et al. 1991; Hirano and Mitchison 1993; Gasser 1995). However, we have detected no genetic interaction between *trf4* and *top2-ts* mutations under conditions where a clear interaction is observed between *top1* and *top2-ts*, nor does *TRF4* affect the level or activity of topo II (Sadoff et al. 1995). Consistent with a model in which *TRF4* function overlaps with *TOP1* and not *TOP2* function, antibody-blocking experiments in the *Xenopus* *in vitro* system indicate that topo II is required for a very early step in condensation (resolution of tangled strands), whereas the *SMC* family members XCAP-C and XCAP-E are required late in the process (actual condensation; for review, see Hirano 1995). In addition, whereas XCAP-C and E are tightly associated with the final condensed chromosome, topo II does not appear to be (Hirano and Mitchison 1994). Thus, it is likely that *TRF4* and *TOP1* function in a separate aspect of condensation from *TOP2*. If the role of topo II is indeed limited to the early resolution of tangled strands, and the later steps in actual condensation generate torsional stress, that stress could

be relieved as easily by the action of a type I DNA topoisomerase, which makes a single-stranded break during the relaxation reaction, as by a type II DNA topoisomerase, which makes a double-stranded break.

Mitotic chromosome condensation and the spindle assembly checkpoint

Recent results suggest strongly that the primary event that activates the spindle assembly checkpoint in *S. cerevisiae* is failure to attach a microtubule to a kinetochore (Wells and Murray 1996). Data from work in mammalian systems suggests this is also true for higher cells (Rieder et al. 1994) and that a lack of tension resulting from failure of chromosomes to attach to kinetochore microtubules from both poles is what is sensed by the checkpoint (Li and Nicklas 1995). The failure to condense chromosomes at mitosis in *top1 trf4-ts* cells at the restrictive temperature may result in the inability of kinetochore microtubules to attach properly to kinetochores, since the *MAD1*-dependent checkpoint is engaged by the resulting defect. It is also possible that the kinetochore itself is not assembled correctly in the *top1 trf4-ts* mutant. However, we consider a defect in kinetochore assembly less likely because the *top1 trf4-ts* mutant is clearly defective in the condensation of noncentromeric (rDNA) chromatin. It also appears that the defect in *top1 trf4-ts* cells results in defective nuclear positioning with respect to the bud site. This may indicate that extra nuclear microtubules required for correct nuclear migration (Eshel et al. 1993) cannot function properly because of a defect in the intranuclear spindle that result from chromosome decondensation.

Whereas the *MAD-BUB*-dependent checkpoint is apparently engaged by the defect in *top1 trf4-ts* cells maintained at the restrictive temperature, a uniform cell-cycle arrest does not result. It could be that initially kinetochore microtubule attachment is defective, which results in cell-cycle delay, but that after longer times that ability of the checkpoint itself to function is impaired by the appearance of decondensed chromosomes. Alternatively, a weak signal could be generated by the resulting defect, which is eventually overridden. Indeed, most cells with activated *MAD1* or *RAD9*-dependent checkpoints do eventually divide (discussed in Wells and Murray 1996). *smc-ts* mutants exhibit a similar catastrophic mitosis that results in a nonuniform terminal arrest morphology that is *RAD9*-independent (Strunnikov et al. 1993), although *MAD1*-dependency was not tested.

Association of Trf4p, Smc1p, and Smc2p

The genetic interaction between a *trf4* and an *smc1* mutant suggests that both genes function in a related pathway. Further supporting this, we find that when Trf4-PYP is expressed from the *GAL* promoter, and the *Smc* proteins are overexpressed from 2 μ plasmids, Smc1-MYCp, Smc2-HAp, and Trf4-PYP can be coimmunoprecipitated. Overexpression is necessary to monitor the interaction because the *Smc* proteins are present in low

abundance (Strunnikov et al. 1995) and Trf4-PYP expressed from its own promoter is not readily detected on Western blots. Nonetheless, because of the requirement for overexpression of these proteins, the physical interaction must be interpreted cautiously. The predicted structure of the SMC gene family shows that each member contains an amino-terminal NTP binding site, a long intervening coiled-coil region with a spacer region of unknown function and a carboxy-terminal putative helix-loop-helix region that may mediate DNA binding (for review, see Peterson 1994; Hirano 1995). The overall structural organization of these proteins is similar to mechanochemical motor proteins such as kinesin. It has been suggested that SMC proteins may bind to and compact DNA via ATP-dependent movement in a manner analogous to the way kinesin promotes movement along microtubules (Strunnikov et al. 1993; Peterson 1994). An alternative model is that SMC heterodimers form a coiled-coil-based filament that serves as a scaffold for condensation (Hirano 1995). Filament formation may be required to form a complete NTP-binding site because one putative NTP-binding-domain motif is found at the amino terminus (Walker A) and another (Walker B) at the carboxyl terminus. *TRF4* is not related to the SMC family and it may promote an independent step in the condensation process via Smc1p/Smc2p association or be required for assembly of the SMC complex. The very high percentage of charged residues in Trf4p (35%) with little overall net charge (18% acidic and 17% basic residues) is characteristic of nonhistone chromosomal proteins (Castaño et al. 1996) and may indicate that Trf4p associates directly with condensed chromatin. The XCAP proteins are also present in a complex with several other as-yet-unidentified proteins (T. Hirano, pers. comm.). Identification of the *Xenopus* *TRF4* homolog would be useful to examine its role in an in vitro system, and, because *TRF4* is highly conserved across species (Castaño et al. 1996), this should be straightforward. Whether topo I is required in the *Xenopus* in vitro system has not been tested (T. Hirano, pers. comm.).

The *S. pombe* genes *cut3* and *cut14* (Saka et al. 1994) are the closest known *S. pombe* homologs of *S. cerevisiae* *SMC1* and *SMC2*, respectively, based on evolutionary comparisons (Strunnikov et al. 1995). Yanagida and colleagues have shown that the *S. pombe* genes are also required for chromosome condensation and proper nuclear division (Saka et al. 1994). Significantly, these authors also note the surprising fact that topo I overexpression is able to suppress the temperature sensitivity of a *cut3-477* mutation, whereas topo II overexpression is not (Saka et al. 1994). In addition *cut3-477 top1* double mutants were observed to be inviable at 36°C (Saka et al. 1994). This suggests that the involvement of topo I in mitotic chromosome structure may be an evolutionarily conserved function, and further supports a model in which topo I activity is involved directly in the condensation process. We have identified clear *S. pombe* and human *TRF4* homologs in data-base searches (Castaño et al. 1996). The *S. pombe* *TRF4*-related gene (GenBank accession no. Z66568) was identified via the genome proj-

ect and the phenotype of a mutation in this gene is not known, although, based on our results with the *S. cerevisiae* homologs, it may show a genetic interaction with *cut3* or *cut14* mutations. We have also isolated an *S. cerevisiae* *TRF4*-related gene, *TRF5*, in a high-copy suppression screen. Trf5p is 55% identical and 72% similar to Trf4p and, like Trf4p, is homologous to the catalytically dispensable amino terminus of *TOP1* (Castaño et al. 1996). The *trf4 trf5* double mutant is inviable and a *trf4-ts trf5* double mutant is defective in nuclear division at the restrictive temperature (Castaño et al. 1996). Thus, *TRF4* is a member of an evolutionarily conserved gene family that functions in combination with *TOP1* to facilitate chromosome condensation at mitosis. Identification of other proteins that interact with Trf4p and the SMC proteins should yield insight into the mechanism of mitotic chromosome condensation and further understanding of this previously unrecognized biological function of topo I.

Materials and methods

Microbial techniques

Standard methods of yeast genetics were performed (Rose et al. 1990). *Escherichia coli* cells (strain DH5 α) were transformed by electroporation (Dower et al. 1988). Small-scale plasmid DNA isolations were made by the boiling method (Holmes and Quigley 1981) or using Qiagen columns (Qiagen, Chatsworth, CA).

Media and growth conditions

Yeast strains were grown routinely in YEP or synthetic complete media lacking the appropriate supplements with a 2% final concentration of glucose (YPD or SC), or galactose (Sherman 1991). To obtain synchronously growing cultures, cells were grown in YPD at 24°C to an A_{600} of 1.5–2. α -factor was added to a final concentration of 25 μ g/ml to arrest cells in G₁, and cultures were incubated for 3 hr at 24°C. Cells were released from the block by washing with 1 volume of sterile water and resuspending in fresh YPD. Half of the culture was incubated at 24°C, the other half at 37°C, and aliquots were removed every 30 min. Nocodazole arrest was performed on exponentially growing cells in YPD by addition of nocodazole to a final concentration of 20 μ g/ml (from a 10 μ g/ml stock solution in DMSO). Cells were then incubated at either 24°C or 37°C for 3 hr.

Plasmid loss assay

Mitotic stability of *CEN* or 2 μ plasmids was measured by determining the fraction of cells in a culture that lost the plasmid when grown under selective conditions (Strunnikov et al. 1993). Cells containing either pRS316 (*CEN URA3*) or pYEp24 (2 μ *URA3*) were grown overnight in SC-Ura at 30°C. Cells were diluted 1:10 in fresh SC-Ura and grown for two more generations; aliquots of these cultures were then diluted in sterile water and plated for single colonies on SC medium and incubated for 2 days at 30°C. After colonies had grown, plates were replica printed to SC-Ura and SC plates. Percent of plasmid loss is expressed as the ratio of the number of colonies that did not grow on SC-Ura to the total number of colonies formed.

Table 6. Yeast strains

Strain	Genotype	Source
CY143	<i>MATa ade2-1 ura3-1 trp1-1 his3-11,15 leu2-3,112 rDNA::URA3</i>	Laboratory collection
CY184	<i>MATa ade2-1 ura3-1 trp1-1 his3-11,15 leu2-3,112 rDNA::ADE2</i>	Sadoff et al. 1995
CY185	<i>MATa Δtop1-7::LEU2 ade2-1 ura3-1 trp1-1 his3-11,15 leu2-3,112 rDNA::ADE2</i>	Sadoff et al. 1995
CY855	<i>MATa Δtrf4-101::HIS3 ade2-1 ura3-1 trp1-1 his3-11,15 leu2-3,112 rDNA::ADE2</i>	Sadoff et al. 1995
CY890	<i>MATa Δtrf4-101::HIS3 ade2-1 ura3-1 trp1-1 his3-11,15 leu2-3,112 rDNA::URA3</i>	This study
CY920	<i>MATa Δtop1-7::LEU2 ade2-1 ura3-1 trp1-1 his3-11,15 leu2-3,112 rDNA::ADE2</i>	This study
CY923	<i>MATa Δtrf4-101::HIS3 Δtop1-7::LEU2 ade2-1 ura3-1 trp1-1 his3-11,15 leu2-3,112 rDNA::ADE2/pCB432</i>	This study
CY938	<i>MATa Δtop1-7::LEU2 trf4-ts1 ade2-1 ura3-1 trp1-1 his3-11,15 leu2-3,112 rDNA::ADE2</i>	This study
CY950	<i>MATa Δtop1-7::LEU2 trf4-ts1 ade2-1 ura3-1 trp1-1 his3-11,15 leu2-3,112 rDNA::ADE2</i>	This study
CY970	<i>MATa Δtop1-7::LEU2 trf4-ts1 mad1Δ2::URA3 ade2-1 ura3-1 trp1-1 his3-11,15 leu2-3,112 rDNA::ADE2</i>	This study
CY978	<i>MATa Δtop1-7::LEU2 trf4-ts1 rad9Δ::URA3 ade2-1 ura3-1 trp1-1 his3-11,15 leu2-3,112 rDNA::ADE2</i>	This study
2bAS283	<i>MATa ade2 his3 leu2 lys2 ura3 smc2-6</i>	Strunnikov et al. 1995
3aAS273	<i>MATa ade2 his3 (leu2) lys2 ura3 smc1-2::LEU2</i>	Strunnikov et al. 1995

Strain constructions

Yeast strains are listed in Table 6. *CY950*: Plasmid pCB695 (*trf4-ts1*) integrating plasmid was partially digested with *EcoRI* and the linear fragment was integrated at the *TRF4* locus in strain *CY185* (*MATa top1-7::LEU2*) by selecting for *Ura*⁺ at 30°C. Integration at the *TRF4* locus was confirmed by Southern analysis. Excision of the plasmid was selected for by streaking cells on plates containing 1mg/ml 5-fluoro-orotic acid (5-FOA) at 24°C. 5-FOA-resistant colonies were then screened for temperature sensitive (ts) growth at 37°C. The resulting ts strain was named *CY938*. The mating type of *CY938* was switched to *a* to generate *CY950* by introducing pCB10, which contains the *HO* gene expressed from the *GAL1* promoter (Herskowitz and

Jensen 1991). After induction by growth in galactose containing medium, colonies were screened to identify *a* maters. *CY890*: Plasmid pCB470 (Sadoff et al. 1995) was digested with *SacI* to release a 4-kb *trf4-101::HIS3* fragment that was used to disrupt the *TRF4* locus in strain *CY143*. Disruption at the *TRF4* locus was confirmed by Southern blot. This strain contains *rDNA::URA3*. *Ura*⁻ derivatives were selected for by streaking on 5FOA plates at 30°C in order to create a selection for *URA3* marked plasmids for immunoprecipitation studies. *CY970*: Plasmid pKH160 containing the *mad1Δ2::URA3* deletion allele (Hardwick and Murray 1995), was digested with *EcoRI* and *HindIII*, and the fragment containing this allele was used to disrupt the *MAD1* locus of strain *CY950*. Disruption of *MAD1*

Table 7. Plasmids

Plasmid	Genotype	Source
pCB10	<i>pGAL::HO URA3 CEN</i>	Herskowitz and Jensen 1991
pCB119	<i>TOP1-HA URA3 2μ</i>	This study
pCB431	<i>TRF4 TRP1 CEN</i>	Sadoff et al. 1995
pCB432	<i>TRF4 URA3 CEN</i>	Sadoff et al. 1995
pCB635	<i>TRF4 TRP1 CEN</i>	This study
pCB694	<i>trf4-ts1 R360C TRP1 CEN</i>	This study
pCB695	<i>trf4-ts1 URA3</i>	This study
pCB699	<i>TRF4-PY URA3 CEN</i>	This study
pCB700	<i>pGAL1::TRF4-PY URA3 LEU2 2μ</i>	This study
pCB787	<i>pGAL1::TRF4-PY URA3 2μ</i>	This study
pRS306	<i>URA3</i>	Sikorski and Hieter 1989
pRS314	<i>TRP1 CEN</i>	Sikorski and Hieter 1989
pRS316	<i>URA3 CEN</i>	Sikorski and Hieter 1989
pYEp24	<i>URA3 2μ</i>	Lab collection
pYEp435	<i>pGAL vector URA3 LEU2 2μ</i>	Ma et al. 1987
pAS258	<i>SMC1-MYC URA3 2μ</i>	Strunnikov et al. 1995
pAS428	<i>SMC2::HA LEU2 2μ</i>	Strunnikov et al. 1995
pKH160	<i>mad1Δ2::URA3</i>	Hardwick and Murray 1995
pRR330	<i>rad9Δ::URA3</i>	Schiestl et al. 1989
p632	<i>rDNA</i>	Keil and Roeder 1984

was confirmed by Southern blot. CY987: plasmid pRR330 was digested with *EcoRI* and *Sall* and the 7.1-kb fragment containing the *rad9Δ::URA3* allele (Schiestl et al. 1989) was used to disrupt the *RAD9* locus of strain CY950. Disruption of *RAD9* was verified by Southern blot.

Plasmid constructions

Plasmids used in this study are listed in Table 7. pCB635: A 2.1-kb PCR product containing wild-type *TRF4* and 396 bp upstream from the ATG was made using primers to which *BglII* sites were added: 5'-GAAGATCTAATATCCTATAAACT-CAGAA-3' and 5'-GAAGATCTTCTTAAACGTATAAGGAT-TAT-3' using pCB431 (*TRF4 TRP1 CEN*; Sadoff et al. 1995) as a template. The PCR product was digested with *BglII* and cloned into the *BamHI* site of pRS314 (Sikorski and Hieter 1989). pCB694: pCB635 was mutagenized in vitro with hydroxylamine (Rose et al. 1990). 10 μg of plasmid DNA was resuspended in 1 M hydroxylamine and incubated at 75°C for 1 hr. After gel filtration in a Sephadex G-50 spin column, transformation of *E. coli* and isolation of plasmid DNA, the pool of mutant plasmids was used to transform CY923 (*trf4-101::HIS3 top1-7::LEU2/pTRF4 URA3 CEN*) by selecting for *Trp*⁺. Transformants were grown nonselectively for the *pTRF4 URA3* plasmid and printed to 5-FOA plates at 24°C and 37°C. Colonies that gave 5-FOA resistant segregants at 24°C but not at 37°C were retained as *trf4-ts* candidates. pCB694 was then recovered from one *trf4-ts* candidate grown at 24°C and it was confirmed that the *trf4 ts* phenotype was plasmid-linked. The entire *TRF4* gene was sequenced from pCB694 and found to contain a single G to A transition mutation, which results in a change of arginine 360 to cysteine (R360C or *ts1*). pCB695: plasmid pCB694 was digested with *KpnI* and *NotI* and the 2.1-kb fragment containing *trf4-ts1* was cloned into pRS306 previously digested with the same enzymes to create a *trf4-ts1 URA3* integrating plasmid. pCB700: a PCR product containing *TRF4* and two tandem copies of the polyoma epitope tag (PY; Grussenmeyer et al. 1985) at the *TRF4* carboxyl terminus was synthesized using the following primers: 5'-TCCCCCGGGGAGATCTATGGGGGCAAA-GAGTG-3' and 5'-GAAGATCTTTAAAGGGTATAAGGATT-TTCCATTGGCATATATTCCATTTCATTCATC-3'. The PCR product was then digested with *BglII* and cloned into the *BamHI* site of pYEp435 to create a *pGALI::TRF4-PY*. pCB787: pCB700 was digested with *KpnI* and *Sall* and the 1.5-kb fragment containing a fragment of the *LEU2* gene was removed. The ends were then made blunt using Klenow enzyme and religated.

Immunoprecipitations and Western blotting

Immunoprecipitations were performed as described (Kaffman et al. 1994), with the following modifications. Cultures were grown in SC medium lacking the relevant supplements, cells were harvested, washed, and resuspended in HSB plus protease inhibitors [45 mM HEPES-KOH (pH 7.5) 150 mM NaCl, 10% glycerol, 1 mM EDTA, 0.5% NP-40, 2 mM DTT, 2 mM benzamide, 1 mM PMSF, leupeptin (1 μg/ml), pepstatin (1 μg/ml), antipain (1 μg/ml), and chymostatin (1 μg/ml)]. Crude protein extracts were made by glass bead lysis (Kolodziej and Young 1991), and 2 mg of total protein was incubated with 10 μl of protein G-Sepharose beads that had been coupled to a monoclonal antibody against a polyoma epitope (α-PY) as described (Harlow and Lane 1988). The immunoprecipitated material was collected by centrifugation and the supernatant was recovered for later analysis. After 4 washes with 2 volumes of PBS [150

mM NaCl, 10 mM NaPO₄ (pH 7.4) + 0.1% NP-40], the samples were resuspended in 10 μl of SDS-PAGE sample buffer containing 2-mercaptoethanol and boiled for 5 min before loading on a 10% SDS polyacrylamide gel. Following electrophoresis, the gel was electrotransferred to nitrocellulose membranes and probed with either affinity-purified α-PY monoclonal antibody from cell line AK2987 (1 to 500 dilution), α-HA monoclonal antibody 12 CA5 (1 to 13000 dilution), or α-MYC (9E10) monoclonal antibody (Santa Cruz Biotechnology; 1:1000 dilution); the secondary antibody used was HRP-coupled-goat antimouse IgG (Bio-Rad laboratories, Richmond CA; 1:10,000 dilution). The blots were developed with enhanced chemiluminescence reagents (Amersham).

Flow cytometry

Yeast cells were synchronized with α-factor for 3 hr at 24°C, collected by centrifugation and resuspended in fresh media with no α-factor at either 24°C or 37°C. Aliquots were taken immediately after pheromone release, and every 30 min thereafter. Cells were fixed with 70% ethanol, treated with 0.1% RNase followed by 5 mg/ml proteinase K prior to staining with propidium iodide. Cells were analyzed on a Beckton Dickinson flow machine.

Indirect immunofluorescence and nuclear staining

We used adaptations of the protocols described (Pringle et al. 1991) as follows: Samples of cultures of synchronously growing cells were fixed in 3.7% para-formaldehyde with gentle shaking for 1 hr at room temperature. Cells were then retained by filtration through a 0.2 μm disposable filter and washed with 3 volumes of solution B [1.2 M sorbitol in 100 mM KPO₄ (pH 7.5)]. Cells were resuspended in 250 μl of solution B and spheroplasted by treatment with lyticase. After washing, the cells were adhered to polyethylenimine-coated slides and fixed by dipping in cold methanol. Slides were incubated with a monoclonal rat antiyeast tubulin antibody (YOL1/34; 1:10 dilution, Accurate Chemical, Westbury, NY). The secondary antibody was Texas Red-conjugated donkey antirat IgG (1:50 dilution; Jackson ImmunoResearch Labs, West Grove, PA). Cells were then stained with 4,6-diamidino-2-phenylindole (DAPI, 0.2 μg/ml) for 15 min, washed and mounted in 0.1% *p*-phenylenediamine in 90% glycerol and phosphate buffered saline (PBS).

FISH assay for rDNA chromosome condensation

Labeling DNA for fluorescence in situ hybridization Thirty nanograms of p632 plasmid DNA containing rDNA (Keil and Roeder 1984; Guacci et al. 1994) was digested with *Sau3A*. The restriction enzyme was removed by extraction with phenol:chloroform 1:1 and the DNA was precipitated with ethanol. The DNA was resuspended in 5 μl water and labeled with biotin-dUTP using the Bionick kit (GIBCO-BRL).

Fluorescence in situ hybridization with rDNA Yeast strains were grown in the presence or absence of nocodazole or α-factor to an A₆₀₀ of 0.5. Fixation and hybridization were carried out as described (Guacci et al. 1994) with the following modifications. One milliliter of cells were fixed by the addition of 100 μl of 37% formaldehyde followed by incubation for 2 hr at room temperature. After washing twice in 1 ml of water, cells were resuspended in 500 μl of a buffer containing 1 M sorbitol, 20 mM potassium phosphate buffer (pH 7.4), 45 mM beta-mercaptoethanol, and 100 μg/ml lyticase. After a 1 hr incubation at 37°C, 500

μ l of 1% Triton was added. The spheroplasted cells were spun down and resuspended in 1 ml of water. Cells (20 μ l) were dropped onto polylysine coated slides and, after 5 min of attachment, washed with 0.5% sodium dodecyl sulfate. Slides were fixed in methanol:acetic acid (3:1) and dried for 24 hr at room temperature before hybridization.

Slides were treated with 100 μ g/ml RNaseA in 2 $\times$ SSC (Sambrook et al. 1989) for 1 hr at 37°C. After dehydration in a 70%, 80%, 95%, 100% ethanol wash series, slides were denatured in 2 $\times$ SSC, 70% formamide (pH 7) at 70°C. Slides were treated with 4mAnson Units/ml proteinase K (GIBCO-BRL) in 20 mM Tris/HCl (pH 7.5) and 2 mM CaCl_2 for 20 min at 37°C. The slides were dehydrated in an ethanol wash series as above. 1 μ l of biotin-labeled DNA was mixed with 1 μ l of human placental DNA (Sigma Chemicals) and 5 μ l of 1.4 $\times$ yeast hybridization mix (1 g dextrane sulfate, 5 ml formamide, 1 ml 20 $\times$ SSC). The solution was denatured at 70°C for 10 min and applied to the slides. After coverslip addition, the slides were incubated in a humid chamber overnight at 30°C.

The next day the slides were washed three times in 2 $\times$ SSC, 60% formamide (pH 7) at 37°C, and once in 0.1 M sodium phosphate buffer (pH 8), 0.03% NP40 (Sigma) at room temperature. Fluorescence staining was performed by incubation with 5 μ g/ml FITC-avidin (Vector Laboratories) in 4 $\times$ SSC, 0.1% Triton X100, 5% BSA for 20 min, followed by three washes in 4 $\times$ SSC, 0.1% Triton X-100 (Sigma), incubation with 5 μ g/ml biotinylated anti-avidin (Vector Laboratories) in 4 $\times$ SSC, 0.1% Triton X100, and 5% BSA for 20 min, followed by three washes in 4 $\times$ SSC, 0.1% Triton X-100 (Sigma), and incubation with 5 μ g/ml FITC-avidin (Vector Laboratories) in 4 $\times$ SSC, 0.1% Triton X100, 5% BSA for 20 min, followed by three washes in 4 $\times$ SSC, 0.1% Triton X-100. Slides were desalted in 0.1 $\times$ SSC and dehydrated in an ethanol wash series. Slides were mounted with 10 μ M DAPI in antifade solution (Johnson and Nogueira-Araiyo 1981) and viewed through a Zeiss Axioplan fluorescence microscope using a 100 $\times$ objective lens.

Acknowledgments

The authors thank Doug Koshland, Alexander Strunnikov, and Vincent Guacci for strains, plasmids, and extensive advice on the FISH chromosome condensation assay. We also thank Kim Arndt, Ira Herskowitz, and Andrew Murray for strains and plasmids and Ira Herskowitz, Andrew Murray, Nikki Levin, David Pellman, and M. Mitchell Smith for critical reviews of the manuscript. We are grateful to Pragati Bakshi for help in preparing the manuscript. I.C. was supported in part by a fellowship from the Organization of American States. This work was supported by National Institutes of Health grant GM46877 to M.F.C.

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Genes Dev. 1996, **10**:

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