

# Rap1p telomere association is not required for mitotic stability of a C<sub>3</sub>TA<sub>2</sub> telomere in yeast

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**Telomeric DNA usually consists of a repetitive sequence: C<sub>1-3</sub>A/TG<sub>1-3</sub> in yeast, and C<sub>3</sub>TA<sub>2</sub>/T<sub>2</sub>AG<sub>3</sub> in vertebrates. In yeast, the sequence-specific DNA-binding protein Rap1p is thought to be essential for telomere function. In a *tlc1h* mutant, the templating region of the telomerase RNA gene is altered so that telomerase adds the vertebrate telomere sequence instead of the yeast sequence to the chromosome end. A *tlc1h* strain has short but stable telomeres and no growth defect. We show here that Rap1p and the Rap1p-associated Rif2p did not bind to a telomere that contains purely vertebrate repeats, while the TG<sub>1-3</sub> single-stranded DNA binding protein Cdc13p and the normally non-telomeric protein Tbf1p did bind this telomere. A chromosome with one entirely vertebrate-sequence telomere had a wild-type loss rate, and the telomere was maintained at a short but stable length. However, this telomere was unable to silence a telomere-adjacent *URA3* gene, and the strain carrying this telomere had a severe defect in meiosis. We conclude that Rap1p localization to a C<sub>3</sub>TA<sub>2</sub> telomere is not required for its essential mitotic functions.**  
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## Introduction

Telomeres, the DNA–protein complexes found at the ends of linear eukaryotic chromosomes, usually consist of a short repeated sequence that is G-rich on the strand running 5′ to 3′ towards the telomere. For example, telomeres in the budding yeast *Saccharomyces cerevisiae* contain about 350 bp of C<sub>1-3</sub>A/TG<sub>1-3</sub> DNA, while the telomere sequence in all vertebrates, including humans, is C<sub>3</sub>TA<sub>2</sub>/T<sub>2</sub>AG<sub>3</sub>. In diverse organisms, including yeast and humans, the G-rich strand of the telomere extends to form a 3′ single-strand overhang (reviewed in Shore, 2001).

Telomeres have several important functions. They promote the complete replication of chromosome ends through the addition of repeated sequences by telomerase, a specialized reverse transcriptase. Telomeres have an essential role in capping chromosome ends, preventing the degradation and end-to-end fusions seen at double-stranded DNA breaks. In *S. cerevisiae*, loss of even a single telomere results in a cell-cycle arrest mediated by the DNA damage checkpoint (Sandell and Zakian, 1993). Cells that recover from this arrest without replacing the missing telomere lose the broken chromosome at a very

high rate (Sandell and Zakian, 1993). In many species, from yeasts to mammals, a gene placed next to a telomere can be reversibly silenced, a phenomenon known as telomere position effect, or TPE (reviewed in Tham and Zakian, 2002). Telomeres also play an important role in chromosome pairing during meiosis. In many species, telomeres cluster early in meiosis, a process known as bouquet formation, which may help align homologous chromosomes (reviewed in Scherthan, 2001). In fission yeast, the telomere-associated proteins Taz1 and Rap1 are required for efficient meiosis (Cooper *et al.*, 1997; Chikashige and Hiraoka, 2001; Kanoh and Ishikawa, 2001). In *S. cerevisiae*, the meiosis-specific telomere-associated protein Ndj1p, promotes chromosome pairing and is required for bouquet formation (Chua and Roeder, 1997; Conrad *et al.*, 1997; Trelles-Sticken *et al.*, 1999).

Several proteins are known to associate with *Saccharomyces* telomeres. Rap1p, an essential protein that binds to duplex *S. cerevisiae* telomeric DNA (Berman *et al.*, 1986; Longtine *et al.*, 1989), is the major *in vivo* telomere binding protein present in 10–20 copies per telomere (Conrad *et al.*, 1990; Wright *et al.*, 1992; Gilson *et al.*, 1993). Rap1p recruits two groups of proteins, the Sir proteins that mediate TPE (Aparicio *et al.*, 1991; Moretti *et al.*, 1994) and the Rif proteins that inhibit both telomerase- and recombination-mediated telomere lengthening (Bourns *et al.*, 1998; Teng *et al.*, 2000). Cdc13p, which binds single-stranded TG<sub>1-3</sub> DNA *in vitro* (Lin and Zakian, 1996; Nugent *et al.*, 1996) and chromosome ends *in vivo* (Bourns *et al.*, 1998; Tsukamoto *et al.*, 2001), is essential because it helps protect chromosome ends from degradation (Garvik *et al.*, 1995). Cdc13p also plays a positive role in regulation of telomerase (Nugent *et al.*, 1996; Qi and Zakian, 2000). *Saccharomyces cerevisiae* telomerase includes the catalytic subunit, Est2p, as well as an RNA subunit, *TLC1*, which has a short template region consisting of the C-strand telomere sequence (reviewed in Nugent and Lundblad, 1998).

Rap1p is also required for transcriptional repression at the silent mating-type loci (Shore *et al.*, 1987) and at telomeres (Liu *et al.*, 1994; Moretti *et al.*, 1994). However, since neither mating-type silencing nor TPE is essential, silencing is therefore not an essential function of Rap1p. Rap1p is also a transcriptional activator at many loci in *Saccharomyces*, binding in the upstream regions of ~5% of yeast genes, which together account for ~37% of all yeast mRNA transcripts (Huet *et al.*, 1985). Since many Rap1p-regulated genes are essential, the role of Rap1p as a transcriptional activator is almost certainly essential.

Rap1p homologs have been identified in many species, including other yeasts (Larson *et al.*, 1994; Chikashige and Hiraoka, 2001; Kanoh and Ishikawa, 2001) and humans (Li *et al.*, 2000). Unlike the budding yeast Rap1p, the *Schizosaccharomyces pombe* and human Rap1p homologs

(spRap1 and hRap1) do not bind DNA directly, but are recruited to the telomere through interactions with telomeric DNA-binding proteins (Li *et al.*, 2000; Chikashige and Hiraoka, 2001; Kanoh and Ishikawa, 2001). In addition, spRap1 and hRap1 have not been shown to have any non-telomeric functions, such as transcriptional activation (Li *et al.*, 2000; Chikashige and Hiraoka, 2001; Kanoh and Ishikawa, 2001). Although *S.pombe rap1<sup>+</sup>* is not essential, a *rap1<sup>-</sup>* mutant has long telomeres, lacks TPE and has several meiotic defects, including loss of telomere clustering, reduced sporulation efficiency and low spore viability (Chikashige and Hiraoka, 2001; Kanoh and Ishikawa, 2001). These phenotypes are indistinguishable from those of a strain lacking Taz1, the telomere-binding protein that recruits spRap1 to the telomere (Cooper *et al.*, 1997, 1998; Nimmo *et al.*, 1998).

*Saccharomyces cerevisiae* strains with *rap1<sup>ts</sup>* alleles that reduce the ability of Rap1p to bind DNA exhibit telomere shortening at semipermissive temperatures (Conrad *et al.*, 1990; Lustig *et al.*, 1990). Moreover, overexpression of Rap1p leads to increased rates of chromosome loss (Conrad *et al.*, 1990). These data suggest that Rap1p is required not only for transcription but also for the stability function of yeast telomeres. However, the widespread role of Rap1p in transcription makes it difficult to use a standard genetic approach to determine if telomeres lacking Rap1p are functional. As an alternative, we altered the telomere sequence itself. A strain in which the yeast telomere sequence was changed to match the vertebrate sequence, the *tlc1-human* or *tlc1h* strain, is viable but has short telomeres (Henning *et al.*, 1998). We constructed a *tlc1h* strain that had one telomere consisting solely of C<sub>3</sub>TA<sub>2</sub>/T<sub>2</sub>AG<sub>3</sub> vertebrate telomeric DNA, a sequence that is not expected to bind Rap1p (Liu and Tye, 1991). As predicted, this telomere bound neither Rap1p nor the Rap1p-associated protein Rif2p, yet its role in chromosome stability was unimpaired. Thus, binding of Rap1p to a C<sub>3</sub>TA<sub>2</sub> telomere is not essential in mitotic cells.

## Results

### **A telomere with only vertebrate repeats can be maintained in yeast**

*TLC1* encodes the RNA subunit of yeast telomerase. The *tlc1h* gene is an allele of *TLC1* in which the templating portion of the gene is modified to encode the vertebrate telomere repeat (Henning *et al.*, 1998). Because only the distal third of yeast telomeres is subject to degradation and telomerase-mediated resynthesis (Wang *et al.*, 1989), even after prolonged growth, telomeres in a *tlc1h* strain consist of a mixture of vertebrate C<sub>3</sub>TA<sub>2</sub>/T<sub>2</sub>AG<sub>3</sub> and yeast C<sub>1-3</sub>A/TG<sub>1-3</sub> repeats. These mixed-sequence telomeres are ~150 bp shorter than wild type (Henning *et al.*, 1998).

Rap1p does not bind the vertebrate C<sub>3</sub>TA<sub>2</sub> telomere sequence *in vitro* (Liu and Tye, 1991). To determine if vertebrate telomeric DNA can supply all telomere functions in yeast, we constructed a strain called 499UT-H (UT for *URA3* at telomere, H for human telomeric DNA) in which the left telomere of chromosome VII bears only C<sub>3</sub>TA<sub>2</sub> repeats, rather than a mixture of vertebrate and yeast repeats. The VII-L telomere in a *tlc1h* strain was replaced with the *URA3* gene and 60 bp of vertebrate

telomere sequence that was lengthened *in vivo* by the addition of C<sub>3</sub>TA<sub>2</sub> repeats (Figure 1A). The wild-type control strain for 499UT-H was 499UT-Y (Y for yeast telomeric DNA), an otherwise isogenic strain that contained a wild-type *TLC1* gene, fully wild-type telomeres and *URA3* adjacent to the VII-L telomere.

To confirm that the VII-L telomere in 499UT-H contained only vertebrate telomeric DNA, telomeres from three independent transformants obtained using the UT-H construct were cloned and sequenced ~125 cell divisions after each received the modified telomere (Figure 1B). The amount of telomeric DNA present at individual VII-L telomeres from the *tlc1h* strain ranged from 210 to 240 bp. For comparison, VII-L telomeres cloned from the control 499UT-Y strain contained 320–370 bp of C<sub>1-3</sub>A DNA. None of the 499UT-H clones contained yeast C<sub>1-3</sub>A repeats but rather had up to 35 pure C<sub>3</sub>TA<sub>2</sub> repeats. The VII-L telomere consisting solely of vertebrate telomeric DNA will hereafter be called the C<sub>3</sub>TA<sub>2</sub> telomere.

### **Replication and length regulation of the C<sub>3</sub>TA<sub>2</sub> telomere**

As reported previously (Henning *et al.*, 1998), the mixed vertebrate and yeast sequence telomeres in a *tlc1h* strain were ~150 bp shorter than telomeres in a *TLC1* wild-type strain (Figure 1C, center and right panels). A similar shortening was seen at the C<sub>3</sub>TA<sub>2</sub> telomere (Figure 1C, left panel). The lengths of both the C<sub>3</sub>TA<sub>2</sub> telomere and the mixed-sequence telomeres were stable over at least 500 cell divisions (Figure 1; data not shown).

Yeast telomeres are normally maintained by telomerase. In addition to *TLC1*, telomerase-mediated telomere lengthening requires at least four genes, including *EST2*, which encodes the catalytic subunit of telomerase (Lendvay *et al.*, 1996; Lingner *et al.*, 1997). In the absence of telomerase, telomeres can be maintained by *RAD52*-dependent homologous recombination, leading to an amplification of either sub-telomeric or telomeric repeats (Lundblad and Blackburn, 1993; Teng and Zakian, 1999). To determine which pathway, telomerase or recombination, was maintaining telomeres in the *tlc1h* strain, heterozygous deletions of *EST2* and *RAD52* were made in a diploid 499UT-H/500UT-H strain (500UT-H is identical to 499UT-H except for being of opposite mating type). The diploid was sporulated, tetrads dissected and spore products of the desired genotypes identified by replica plating.

Viability and telomere length in the 499UT-H strain were unaffected by deletion of the homologous recombination protein *RAD52* (Figure 1D). In contrast, no viable *est2 tlc1h* spores were recovered (data not shown). The synthetic lethality observed for the *est2 tlc1h* strain was probably due to the fact that telomeres in the *tlc1h/tlc1h* strain were shorter than in a wild-type strain. Strains lacking an *EST* gene in an otherwise wild-type background senesce after 50–100 divisions (Lundblad and Szostak, 1989). Since the *tlc1h* strain started with telomeres that were ~150 bp shorter than the wild-type length, telomerase-deficient *tlc1h* cells should senesce after ~25–50 generations. Since it takes ~25 generations for a spore to generate a colony, even those *est2 tlc1h* spores that were able to form a colony after germination would

senescence upon the replica plating that was used to identify *est2Δ* spores. We conclude that the  $C_3TA_2$  telomere is maintained by telomerase, not recombination.

### The $C_3TA_2$ telomere does not increase the loss rate of chromosome VII

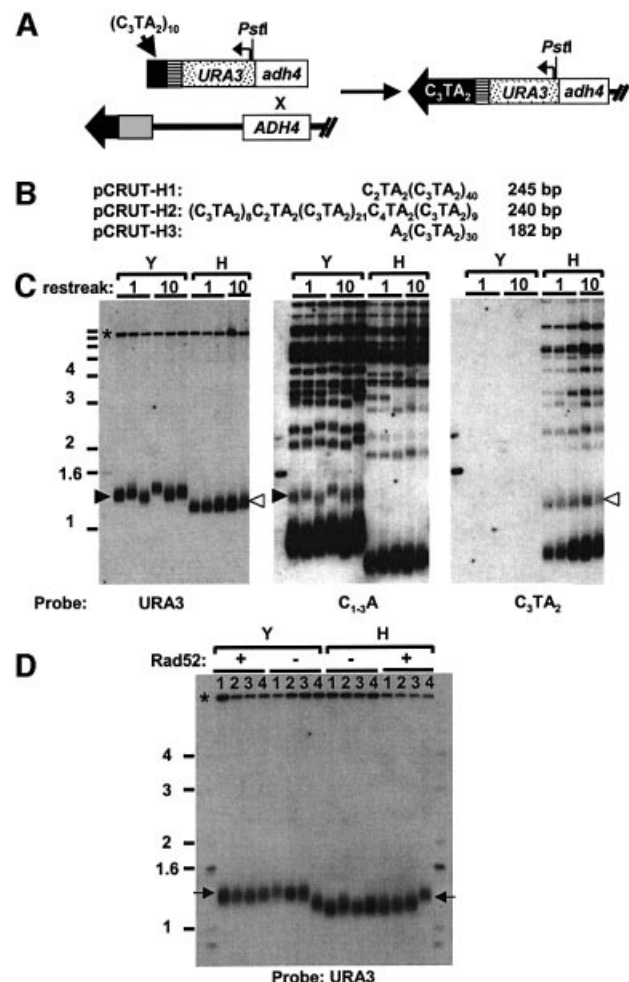
Removal of the yeast VII-L telomere results in a dramatic increase in the rate at which this chromosome is lost (Sandell and Zakian, 1993). Although the growth rate of the *tlc1h* strain with the  $C_3TA_2$  telomere was indistinguishable from wild type, loss of a single chromosome must increase by  $\sim 1000$ -fold before it affects the overall growth rate of a strain. To determine if the  $C_3TA_2$  telomere affects the mitotic stability of chromosome VII, the loss rate of this chromosome was measured using fluctuation analysis in a *tlc1h* strain that was disomic for chromosome VII, but had only one copy of each of the other 15 yeast chromosomes (Figure 2A). One copy of chromosome VII carried the  $C_3TA_2$  telomere while the other copy and all other telomeres in the cell had mixed-sequence telomeres. Whereas the original disome strain produced red colonies that die on cycloheximide plates, cells that lost the chromosome with the  $C_3TA_2$  telomere generated white, cycloheximide-resistant colonies. As a control, we also determined the loss rate of chromosome VII from an otherwise isogenic *TLC1* strain, with completely wild-type telomeres. This analysis revealed that the loss rate of chromosome VII with the  $C_3TA_2$  telomere was statistically indistinguishable from the loss rate of chromosome

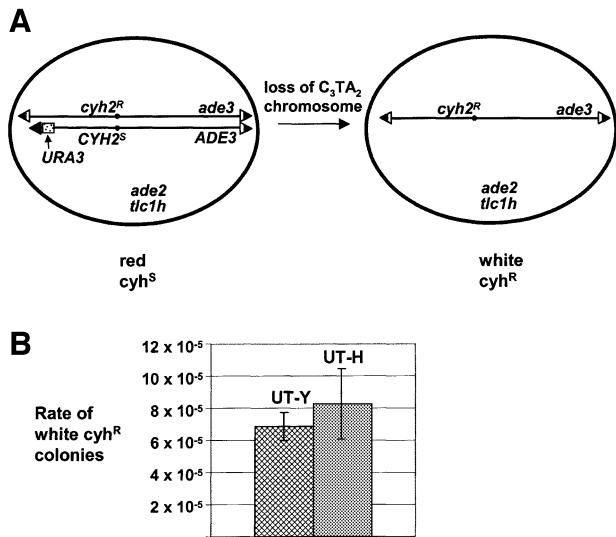
VII in a *TLC1* strain:  $\sim 7-8 \times 10^{-5}$  losses per cell division (Figure 2B). The rate of mitotic recombination on the VII-L disome can be determined by counting the number of red, cycloheximide-resistant colonies that have recombined between the *CYH2* and *ADE3* markers on opposite arms of chromosome VII. This rate was  $< 10^{-5}$  in both the *tlc1h* strain with the  $C_3TA_2$  telomere and the *TLC1* wild-type control. Thus, the stability of the chromosome with the  $C_3TA_2$  telomere was not due to it having increased mitotic recombination.

### The $C_3TA_2$ telomere does not silence expression of an adjacent *URA3* gene

TPE in wild-type yeast is the phenomenon where transcription of genes next to telomeres is reversibly repressed (Gottschling *et al.*, 1990). TPE is often monitored by determining the fraction of cells with a telomeric *URA3* gene that are able to grow on medium containing 5-fluoro orotic acid (FOA), a compound that kills cells expressing *Ura3p*. To determine if the  $C_3TA_2$  telomere exhibited TPE, 10-fold serial dilutions of the 499UT-H strain were spotted on plates containing FOA. The same dilutions were also plated on complete medium lacking FOA (YC plates) to monitor viability. While the *TLC1* 499UT-Y strain grew well on FOA plates, almost no growth was observed for the *tlc1h* 499UT-H strain

**Fig. 1.** A telomere consisting solely of vertebrate telomeric DNA can be stably maintained in yeast. (A) To generate a completely vertebrate-sequence telomere at chromosome VII-L, *EcoRI-Sall*-digested pUT-H was integrated at the *ADH4* locus on chromosome VII-L in a *tlc1h* strain, in a manner that deletes the terminal  $\sim 20$  kb from the chromosome (Gottschling *et al.*, 1990). The 60 bp  $C_3TA_2$  sequence (black box) acts as a seed for telomere formation. The *tlc1h* telomerase can add only vertebrate repeats to this end, resulting in a VII-L telomere that contains no yeast telomeric repeats. A unique sequence (striped block) between the telomere seed and the *URA3* gene was used as a PCR primer site for telomere sequencing and chromatin immunoprecipitations. The arrow above the spotted box indicates the *URA3* promoter. The indicated *PstI* site is located upstream of the *URA3* start codon. (B) Sequencing results for modified VII-L telomere. VII-L telomeres from the 499UT-H strain were cloned and sequenced. Clones were made  $\sim 125$  cell divisions after transformation in three independent integrants of the UT-H construct. Representative sequences for three clones, one from each integrant, are shown; the centromere-proximal end of the sequence is to the right. Seven modified telomeres were sequenced; none contained yeast telomeric DNA. While none of the sequenced telomeres contained the heterogeneous  $C_{1-3}A$  repeats characteristic of yeast telomeres, the VII-L telomere in the 499UT-H2 transformant contained one copy each of two variants of the vertebrate repeat:  $C_4TA_2$  and  $C_2TA_2$ . The 499UT-H1 strain, with 245 bp of pure vertebrate repeats, was used for all subsequent experiments. (C) Telomere length is stable in the 499UT-H strain over many generations. Telomere length in the 499UT-Y and 499UT-H strains was assayed after one and ten serial restreaks on YC plates. Genomic DNA was digested with *PstI* and *XhoI*; the Southern blot was probed sequentially with *URA3*,  $C_{1-3}A$  and  $C_3TA_2$ . The upper band in the *URA3*-probed blot (\*) is the endogenous *ura3-52* allele. Two to three independent colonies from each strain are shown. Black arrowheads indicate the 499UT-Y VII-L telomere; white arrowheads indicate the 499UT-H  $C_3TA_2$  VII-L telomere. (D) Effect of *rad52Δ* on telomere length. Southern blots of *PstI*-digested genomic DNA from four serial restreaks of *rad52* and *RAD52* versions of the 499UT-Y and 499UT-H strains were probed with *URA3* which detects the VII-L telomere fragment. The upper band in the *URA3*-probed blot (\*) is the endogenous *ura3-52* allele. Arrows indicate the VII-L (*URA3* probe) telomere band.





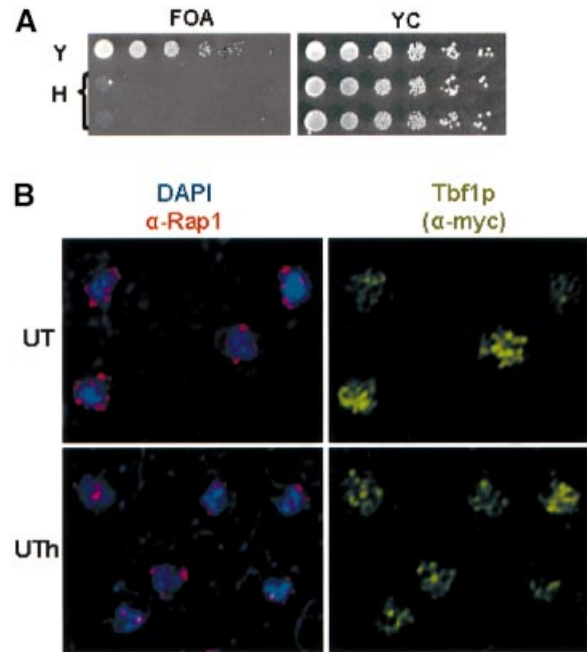
**Fig. 2.** The C<sub>3</sub>TA<sub>2</sub> telomere has a wild-type loss rate. (A) Disome strain for chromosome stability assay. Loss of the test copy of chromosome VII was measured in a chromosome VII disome strain with the *tlc1h* mutation by selecting for white, cycloheximide-resistant colonies. *URA3* with a vertebrate telomere seed was integrated at the VII-L telomere on the test copy of chromosome VII, generating a telomere containing solely vertebrate telomeric DNA. The control strain was a VII-L disome with *URA3* at the VII-L telomere on the test copy of the chromosome and wild-type *TLC1* (Sandell and Zakian, 1993). C<sub>3</sub>TA<sub>2</sub> repeats are in black portion of triangles; C<sub>1-3</sub>A DNA is in white. (B) Rates of chromosome loss. The average rates of chromosome loss for UT-Y (*TLC1*, wt telomeres) and UT-H (*tlc1h*, C<sub>3</sub>TA<sub>2</sub> VII-L telomere) disome strains. Error bars are standard deviation.

(Figure 3A). This loss of silencing was quantitated by comparing plating efficiency on complete medium versus FOA plates. The frequency of silencing at the C<sub>3</sub>TA<sub>2</sub> telomere was reduced ~30 000-fold, from  $0.54 \pm 0.24$  (average  $\pm$  standard deviation) in the *TLC1* 499UT-Y strain to  $1.8 \pm 0.59 \times 10^{-5}$  at the C<sub>3</sub>TA<sub>2</sub> telomere (Figure 3A).

#### The *tlc1h* strain has a severe meiotic defect

To determine the behavior of the C<sub>3</sub>TA<sub>2</sub> telomere in meiosis, we sporulated a diploid strain that was homozygous for *tlc1h* and the C<sub>3</sub>TA<sub>2</sub> telomere (H/H strain) and compared it with an otherwise isogenic *TLC1/TLC1* diploid that also had *URA3* at the left telomere on both copies of chromosome VII but had fully yeast sequence telomeres (Y/Y strain) (Figure 4A). We also sporulated a *tlc1h/tlc1h* diploid that had one copy of the C<sub>3</sub>TA<sub>2</sub> telomere (H/YH strain; the rest of the telomeres were mixed-sequence telomeres) and a *tlc1h/tlc1h* diploid in which neither of the VII-L telomeres was modified by insertion of *URA3* (YH/YH strain; all telomeres were a mixture of yeast and vertebrate repeats).

Although the frequency of sporulation, as measured by comparing the number of asci with the total number of asci plus unsporulated cells, was only 27% in the H/H strain (compared with 41% for the Y/Y wild-type strain), this difference was not statistically significant (Figure 4B). A much more severe defect was seen when the percentages of asci containing complete tetrads were compared between the two strains; while 82% of asci in the Y/Y wild-type diploid contained four spores, only 11% did so

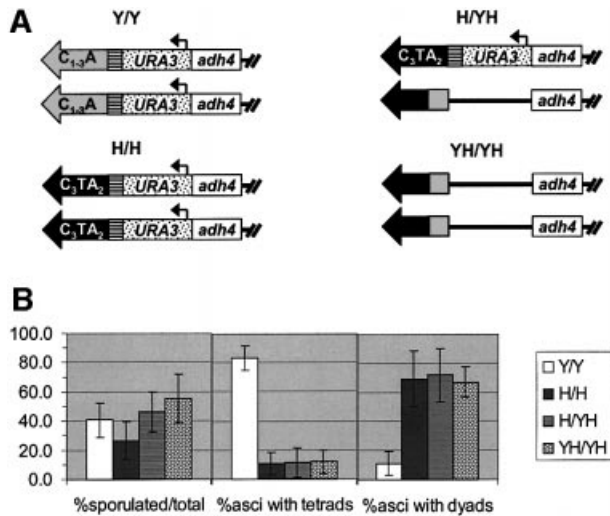


**Fig. 3.** TPE and nuclear organization of a *tlc1h* strain. (A) TPE of 499UT-Y and 499UT-H strains. Ten-fold serial dilutions of 499UT-Y (Y) and 499UT-H (H) were plated on YC plates (to control for viability) and FOA plates (to select for cells that did not express the telomeric *URA3* gene). TPE was also quantitated by comparing plating efficiency of the 499UT-Y and 499UT-H strains on FOA and non-selective medium. (B) Localization of myc-Tbf1p and Rap1p by immunofluorescence. 499UT-Y (Y) and 499UT-H (H) strains expressing myc-tagged Tbf1p were stained with  $\alpha$ -Rap1p polyclonal antibodies and DAPI stain or with  $\alpha$ -myc monoclonal antibodies.

in the H/H strain. Most asci in the H/H strain, 69%, contained only two spores. A similarly low fraction of four-spore asci and high fraction of dyads was seen in the H/YH and YH/YH strains, in which all telomeres contained a mixture of C<sub>3</sub>TA<sub>2</sub> and C<sub>1-3</sub>A telomeric DNA. The viability of spores from either the rare tetrads (66% spore viability) or the more common dyads (64% viability) was modestly reduced in the H/H strain compared with wild type (90% spore viability). Spore viability was similarly reduced in H/YH and YH/YH dyads (46 and 55% viability, respectively).

#### Protein composition of the C<sub>3</sub>TA<sub>2</sub> telomere: Cdc13p and Tbf1p but not Rap1p or Rif2p are bound in vivo

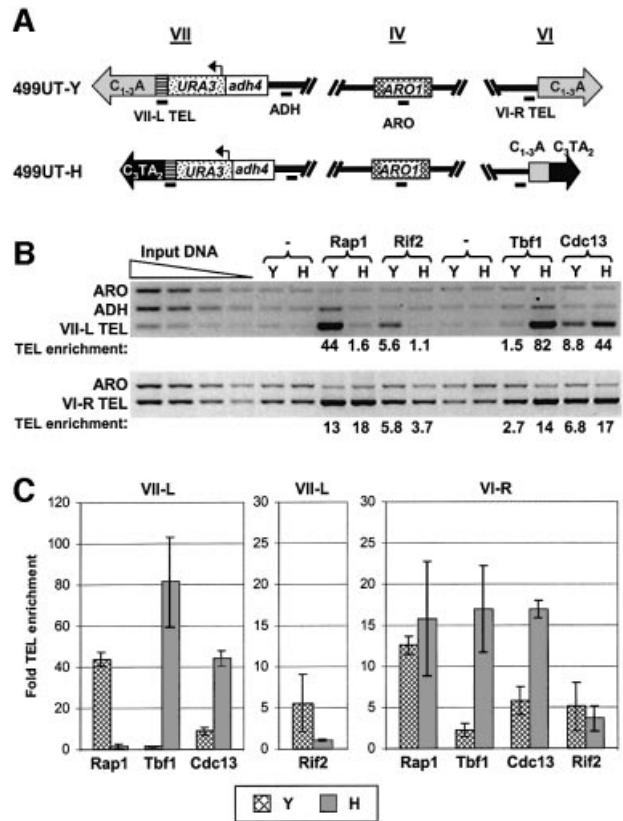
To determine which proteins bound the C<sub>3</sub>TA<sub>2</sub> telomere *in vivo*, we used chromatin immunoprecipitation (ChIP) (Figure 5). In this assay, proteins are crosslinked to each other and to DNA using formaldehyde. The crosslinked DNA is sheared into small pieces by sonication, and precipitated using antibodies to the protein of interest. The crosslinks are then reversed, the DNA is deproteinized, and the precipitated DNA is PCR amplified. To examine whether telomeric sequences were preferentially associated with the proteins of interest, we used a multiplex PCR strategy that was developed to examine telomere-associated proteins (Tsukamoto *et al.*, 2001; Taggart *et al.*, 2002). Four sequences were PCR amplified from the immunoprecipitated DNA (Figure 5A): ARO, a 372 bp



**Fig. 4.** The *tlc1h* strain has altered meiosis. (A) Diploid strains used in meiosis experiments. Diploid strains containing *URA3* on both copies of chromosome VII-L with either solely yeast telomere sequence and wild-type *TLC1* (Y/Y) or homozygous for both the  $C_3TA_2$  telomere and the *tlc1h* mutation (H/H), as well as diploids homozygous for the *tlc1h* mutation and carrying *URA3* with the  $C_3TA_2$  telomere on only one (H/YH) or neither (YH/YH) copy of chromosome VII-L were sporulated (Y indicates solely yeast repeats at VII-L telomere; H indicates solely human telomeric repeats on the VII-L telomere; YH indicates a mixture of yeast and human telomeric DNA at the VII-L telomere; the rest of the telomeres in the *tlc1h* strains were a mixture of yeast and  $C_3TA_2$  repeats). Yeast  $C_{1-3}A$  telomeric repeats are in gray; vertebrate  $C_3TA_2$  telomeric repeats are in black. (B) The number of asci was divided by the number of asci plus unsporulated cells to give the percent sporulated cells. The number of spores per ascus was also determined, and used to calculate the percentage of asci containing dyads and tetrads. Graphs display averages  $\pm$  standard deviation.

segment of *ARO1*, a gene on chromosome IV that is far from either telomere and serves as a negative control for telomere binding; *ADH*, a 301 bp fragment from within *ADH4*, a sub-telomeric gene that is 5 kb from the left telomere of chromosome VII; VII-L TEL, a 252 bp sequence that is immediately adjacent to the modified VII-L telomere; and VI-R TEL, a 265 bp sequence adjacent to the VI-R telomere.

Rap1p and Rif2p, a Rap1p-interacting protein involved in telomere length regulation (Wotton and Shore, 1997), were precipitated using polyclonal sera. Tbf1p, an essential yeast protein of unknown function, binds to duplex  $C_3TA_2$  DNA *in vitro* (Liu and Tye, 1991). Cdc13p, a known *in vivo* single-stranded  $TG_{1-3}$  DNA binding protein (Bourns *et al.*, 1998; Taggart *et al.*, 2002), also binds single-stranded  $T_2AG_3$  DNA *in vitro*, albeit with  $\sim 10$ -fold lower affinity (Lin and Zakian, 1996; Nugent *et al.*, 1996). The endogenous *TBF1* locus was tagged with nine tandem Myc epitopes, while Myc-tagged Cdc13p was expressed under its own promoter from a centromere plasmid. The Myc-tagged proteins were precipitated with  $\alpha$ -myc monoclonal antibodies. The fold TEL enrichment was determined by first normalizing each TEL band to the *ARO* control band, and then comparing the normalized TEL band for each sample with a negative control immunoprecipitated with a non-telomeric polyclonal antibody (for Rap1p and Rif1p samples) or from an untagged strain using  $\alpha$ -myc monoclonal antibody (for the Tbf1p and Cdc13p samples).



**Fig. 5.** Protein composition of the  $C_3TA_2$  telomere. (A) Sequences assayed in ChIPs. Sequences amplified by PCR are indicated by a black bar below the chromosome. Multiplex PCR was performed on the immunoprecipitated samples as well as input DNA dilutions, amplifying four sequences: the internal control sequence *ARO*, the sub-telomeric sequence *ADH* and the telomere-adjacent sequences VII-L TEL and VI-R TEL (representing the completely vertebrate VII-L telomere and the mixed-sequence VI-R telomere, respectively). (B) ChIP results. ChIPs were performed using polyclonal antibodies to Rap1p and Rif2p, and myc-tagged Tbf1p and Cdc13p. An N-terminal 9-myc tag was integrated at the chromosomal copy of Tbf1p; the tagged protein was expressed from the endogenous *TBF1* promoter. Since myc-TBF1 cells grew nearly as well as wild-type, the tagged protein fulfilled the essential function of Tbf1p. Cdc13p with a C-terminal 9-myc tag was expressed from the *CDC13* promoter on a centromere plasmid in a strain that also expressed wild-type Cdc13p from the endogenous locus; when the 9-Myc-Cdc13p is the only Cdc13p in the cell, it supports wild-type telomere length and growth rate (Taggart *et al.*, 2002). Input DNA represents the total chromatin isolated, before immunoprecipitation; two-fold serial dilutions of the input DNA were amplified by PCR to establish the linear range of the PCR amplifications. Negative control samples were immunoprecipitated from untagged strains with either polyclonal antibodies to the non-telomere protein Ypt1 (this was used to define background levels for the polyclonal Rap1p and Rif2p sera) or  $\alpha$ -myc monoclonal antibodies (the control for the myc-Tbf1 and Cdc13-myc strains). (C) Quantitation of ChIP data. The fold enrichment of the VII-L and VI-R TEL bands over background levels are shown; error bars indicate standard deviations. The fold enrichment was normalized to the *ARO1* control band. Note that because the fold enrichments of Rap1p, Tbf1p and Cdc13p at the VII-L telomere are considerably higher than the other signals obtained, the leftmost plot is on a different scale than the center and right plots.

When crosslinked chromatin from the *TLC1* 499UT-Y strain was immunoprecipitated with  $\alpha$ -Rap1p polyclonal antibodies, a strong enrichment ( $44 \pm 3.5$ -fold; average  $\pm$  standard deviation) of the VII-L telomere-adjacent TEL sequence was observed (Figure 5B and C). In contrast, no significant enrichment of the VII-L TEL band was observed at the  $C_3TA_2$  telomere ( $1.6 \pm 1.0$ ) in the

499UT-H strain. To confirm the Rap1p result, the Rap1p-interacting factor Rif2p was also immunoprecipitated. In the *TLC1* strain, Rif2p co-immunoprecipitated the VII-L TEL DNA ( $5.6 \pm 3.5$ -fold enrichment), confirming earlier work using a one-hybrid assay which showed that Rif2p interacts with wild-type telomeres *in vivo* (Bourns *et al.*, 1998). Consistent with the Rap1p results, the C<sub>3</sub>TA<sub>2</sub> VII-L TEL was not precipitated by the  $\alpha$ -Rif2p serum ( $1.1 \pm 0.16$ ). The inverse result was obtained when precipitating myc-Tbf1p. The C<sub>3</sub>TA<sub>2</sub> VII-L TEL band was enriched  $82 \pm 22$ -fold in the 499UT-H strain, but was at background levels ( $1.5 \pm 0.30$ ) in the 499UT-Y strain that contained only yeast telomeric DNA. In contrast, Cdc13p-myc co-precipitated the VII-L TEL DNA in both the 499UT-Y and 499UT-H strains, although the enrichment was even greater in the 499UT-H strain ( $44 \pm 3.8$ -fold) than in the wild-type control ( $8.8 \pm 1.9$ -fold). For each protein, the telomere association was dependent on *in vivo* crosslinking, demonstrating that these associations occurred *in vivo* (data not shown). The enrichments for Rap1p in 499UT-Y and Tbf1p in 499UT-H are minimal estimates, as these TEL bands were outside the linear range for quantitation.

In the 499UT-H strain, the VI-R telomere contained a mixture of vertebrate and yeast telomeric DNA, and thus was expected to bind Rap1p, Rif2p, Cdc13p and Tbf1p. Consistent with this expectation, Cdc13p and Tbf1p bound well to both the VI-R and VII-L telomeres in the 499UT-H strain (Figure 5B and C). In contrast to the results with the C<sub>3</sub>TA<sub>2</sub> telomere, Rap1p and Rif2p both localized to the VI-R telomere in the 499UT-H strain.

#### **Nuclear localization of Rap1p and Tbf1p is unchanged in a *tlc1h* strain**

Immunolocalization reveals that several proteins needed for telomeric silencing, including Rap1p, are concentrated in 6–8 foci that localize to the nuclear periphery (Gotta *et al.*, 1996). *In situ* hybridization shows that many telomeres localize to these Rap1p foci (Gotta *et al.*, 1996). In contrast, Tbf1p has a punctate staining throughout the yeast nucleus (Koering *et al.*, 2000). Given the altered telomere binding of Rap1p and Tbf1p in the *tlc1h* strain, we determined if the nuclear localization of either protein was also affected. To ensure that telomeres had high levels of vertebrate telomere repeats, the *tlc1h* strain was examined after >150 cell divisions of growth. Localization of Rap1p and Tbf1p in nuclei was analyzed by immunofluorescence using either an affinity purified anti-Rap1p polyclonal serum or anti-myc serum (Figure 3B). As reported previously (Koering *et al.*, 2000), Tbf1p exhibited punctate staining in the *TLC1* strain, a pattern that was unaltered in the *tlc1h* strain (Figure 3B, right). Rap1p was concentrated in foci in both the *TLC1* and *tlc1h* strains (Figure 3B, left panels). This analysis demonstrates that the nuclear organization of Rap1p foci, and by inference of telomeres, is not grossly altered in the *tlc1h* strain.

#### **Discussion**

The essential protein Rap1p was thought to be critical both to protect chromosome ends from degradation (Conrad *et al.*, 1990; Lustig *et al.*, 1990) and, in concert with the

associated Rif proteins, for telomere length homeostasis (Marcand *et al.*, 1997). We show here that a telomere consisting solely of C<sub>3</sub>TA<sub>2</sub>/T<sub>2</sub>AG<sub>3</sub> repeats (Figure 1B) did not bind Rap1p or Rif2p (Figure 5). Nonetheless, the length of this telomere was stable for >500 cell divisions (Figure 1C), suggesting that yeast also has a Rap1p-independent mechanism for monitoring telomere length. The absence of Rif proteins, which limit telomerase action (Teng *et al.*, 2000), is normally associated with telomere lengthening (Hardy *et al.*, 1992; Wotton and Shore, 1997). The fact that the C<sub>3</sub>TA<sub>2</sub> telomere that lacked Rif2p (and presumably Rif1p) stabilized at a length shorter than that of a wild-type telomere suggests that Rap1p does help protect telomeres from degradation.

Remarkably, a chromosome with the C<sub>3</sub>TA<sub>2</sub> telomere had a wild-type loss rate (Figure 2). Since loss of only one telomere is sufficient to dramatically destabilize chromosome VII (Sandell and Zakian, 1993), the C<sub>3</sub>TA<sub>2</sub> telomere must be able to supply the critical telomere functions that ensure the stable maintenance of chromosomes. A wild-type telomere is estimated to bind 10–20 Rap1p molecules (Conrad *et al.*, 1990; Wright *et al.*, 1992; Gilson *et al.*, 1993). The VII-L telomere with wild-type telomeric DNA was enriched ~30-fold more than the C<sub>3</sub>TA<sub>2</sub> telomere in the anti-Rap1p immunoprecipitate (Figure 5), suggesting that Rap1p did not bind the C<sub>3</sub>TA<sub>2</sub> telomere *in vivo*. Although we cannot rule out the possibility that the C<sub>3</sub>TA<sub>2</sub> telomere sometimes bound one or a very few Rap1p molecules, our data suggest that the stability functions of yeast telomeres may not require Rap1p. In contrast, both Cdc13p and Tbf1p bound the C<sub>3</sub>TA<sub>2</sub> telomere efficiently (Figure 5). Although no sequence or functional similarities have been shown between Rap1p and Tbf1p outside of the Myb-like DNA binding domain (Bilaud *et al.*, 1996), Tbf1p may provide the stability functions of Rap1p at the modified telomere.

Although the C<sub>3</sub>TA<sub>2</sub> telomere did not impair chromosome stability, meiosis was compromised in the *tlc1h* strain, as demonstrated by a paucity of four spore tetrads (Figure 4B). Because Rap1 is required for efficient meiosis in *S.pombe* (Chikashige and Hiraoka, 2001; Kanoh and Ishikawa, 2001), it is tempting to speculate that the meiotic defect of the *tlc1h* strain is due to the lack of Rap1p at the C<sub>3</sub>TA<sub>2</sub> telomere. However, by the criterion of ChIP, Rap1p bound as well to the mixed-sequence VI-R telomere as it did to telomeres consisting solely of yeast telomeric DNA (Figure 5). Since the YH/YH diploid, with its mixture of yeast and vertebrate telomeric DNA at all telomeres, was as defective in meiosis as the H/H strain that was homozygous for the C<sub>3</sub>TA<sub>2</sub> telomere (Figure 4B), the meiotic defect of the *tlc1h* strain can not be attributed solely to the absence of Rap1p at the VII-L telomere. Ndj1p is a meiosis-specific telomere protein that affects homolog pairing and telomere clustering (Chua and Roeder, 1997; Conrad *et al.*, 1997; Trelles-Sticken *et al.*, 2000). Although we did not examine Ndj1p binding in the *tlc1h* strain, an *ndj1Δ* strain does not have a preponderance of two-spore asci. Thus, even if Ndj1p were improperly localized in the *tlc1h* strain, it would not be sufficient to explain the meiotic defects in this strain. Tbf1p bound well to both the C<sub>3</sub>TA<sub>2</sub> telomere and the mixed-sequence VI-R telomere (Figure 5). Since Tbf1p is not normally telomere bound (Figure 5), perhaps its presence perturbs one or



Table I. Primer sequences

UT-H-5'	CGCGGATCCGGACG(T <sub>2</sub> AG <sub>3</sub> ) <sub>10</sub> GAATTCGG
UT-H-3'	CCGGAATTC(C <sub>3</sub> TA <sub>2</sub> ) <sub>10</sub> CGTCCGGATCCGCG
UT(+)	TCCGGATCCAGAAAAGCAGGCTGGGATATC
UT-H(-)	GGATATCCCAGCCTGCTTTTCTGGATGCGG
TBF1-N1	CCACGGAATTCGTAGAAGCCCGGCGAGACC
TBF1-N2	GGGCACTTGCGAATCACTAGTCATGACAAATGGGGAAAGAAG
TBF1-N3	CACCTCTTTCCCATTTGTCATGACTAGTGATTCCGCAAGTGC
TBF1-N4	CCACGGAGCTCTTAGCTTAGGAAAGCCGTCTGGTCC
est2HISa	ATGAAAATCTTATTTCGAGTTCATTCAGACAAGCTTGACATCGTTCAGAATGACACG
est2HISb	AGCATCATAAGCTGTCAGTATTTTCATGTATTATTAGTACTCTCTGGCCTCCTCTAG

more aspects of meiotic telomere behavior, even in strains that have some C<sub>1-3</sub>A (as well as C<sub>3</sub>TA<sub>2</sub>) telomeric repeats.

TPE was abolished at the C<sub>3</sub>TA<sub>2</sub> telomere (Figure 3A). Tbf1p, which bound efficiently to the C<sub>3</sub>TA<sub>2</sub> telomere, is proposed to halt the spread of telomeric silencing within sub-telomeric DNA (Fourel *et al.*, 1999). The absence of Rap1p, which recruits the Sir silencing proteins to the telomere (Liu *et al.*, 1994; Moretti *et al.*, 1994), and the presence of Tbf1p are sufficient to explain the loss of TPE at the C<sub>3</sub>TA<sub>2</sub> telomere (Figure 3A).

Although Cdc13p binds both single stranded TG<sub>1-3</sub> and T<sub>2</sub>AG<sub>3</sub> DNAs *in vitro*, its affinity for the vertebrate telomeric sequence is considerably lower (Lin and Zakian, 1996; Nugent *et al.*, 1996). Nonetheless, in the *tlc1h* strain, Cdc13p bound well to both the C<sub>3</sub>TA<sub>2</sub> and the mixed-sequence VI-R telomere. In fact, the telomeric fragments from both the VII-L C<sub>3</sub>TA<sub>2</sub> telomere and the mixed-sequence VI-R telomere were enriched by ~5-fold more in the anti-Cdc13p precipitates from the *tlc1h* strain than from a *TLC1* strain that had solely yeast telomeric DNA. The higher telomere enrichment in the anti-Cdc13p immunoprecipitate might reflect an altered telomere structure in the *tlc1h* strain, such as longer or constitutive single-stranded TG<sub>1-3</sub> tails that results in more telomere-bound Cdc13p, or an altered telomeric chromatin, that results in more efficient precipitation of telomere-bound Cdc13p. Although Cdc13p clearly associated with the C<sub>3</sub>TA<sub>2</sub> telomere, the *tlc1h* strain was particularly sensitive to changes in Cdc13p. For example, although replacing the chromosomal *CDC13* gene with the Myc-tagged *CDC13* was readily accomplished in a *TLC1* strain (Tsukamoto *et al.*, 2001), we were unable to construct a *tlc1h* strain expressing only Myc-tagged Cdc13p. To circumvent this problem, we expressed Myc-tagged Cdc13p from a centromeric plasmid in a *CDC13* background.

In summary, although Rap1p has been thought to play a central role in organizing the telomeric chromatin structure in *S.cerevisiae*, surprisingly, telomere binding of Rap1p is not required for the stability functions of a C<sub>3</sub>TA<sub>2</sub> telomere during mitotic growth. A strain carrying an entirely vertebrate-sequence telomere that did not bind Rap1p had no growth defect and no increase in chromosome loss. Although the C<sub>3</sub>TA<sub>2</sub> telomere was somewhat shorter than a wild-type telomere, its length was constant over many generations, indicating that Rap1p is also not essential for regulating telomere length. Consistent with our data, Brevet *et al.* (2003) found that a fully C<sub>3</sub>TA<sub>2</sub> telomere could be maintained indefinitely, did not bind

Rap1p by ChIP, and did not elongate in *rap1t*, *rif1* or *rif2* strains, as predicted if Rap1p did not bind this telomere (V.Brevet and E.Gilson, personal communication). These data suggest that transcriptional activation may be the only essential function of Rap1p, while Cdc13p and its associated proteins Stn1p and Ten1p (Grandin *et al.*, 1997, 2001; Pennock *et al.*, 2001) may suffice for the essential function of telomeres in end protection.

## Materials and methods

### Plasmids and strains

pUT-H was constructed by excising 81 bp of yeast telomere sequence from pADH4UCA III mod(-) (Tsukamoto *et al.*, 2001) and replacing it with 60 bp of vertebrate telomere sequence, obtained by annealing the UT-H-5' and UT-H-3' oligos (Table I), which consist of the sequence (T<sub>2</sub>AG<sub>3</sub>)<sub>10</sub> or (C<sub>3</sub>TA<sub>2</sub>)<sub>10</sub> flanked by *Bam*HI and *Eco*RI sites. The pRS304-myc-TBF1 plasmid (constructed using the method of Michaelis *et al.*, 1997) was used to integrate a 9-myc epitope tag at the N-terminus of the chromosomal copy of *TBF1*. An ~200 bp region upstream of the *TBF1* gene that included the start codon was PCR amplified using primers TBF1-N1 and TBF1-N2 (Table I), which introduced an *Eco*RI site at the 5' end and a *Spe*I site following the start codon. Another 200 bp from the beginning of the *TBF1* gene was PCR amplified with primers TBF1-N3 and TBF1-N4 (Table I), which introduced a *Spe*I site following the start codon and a *Sac*I site at the end of the fragment. These two PCR products, which share 42 bp of sequence around the *TBF1* start codon, were used as the template for a PCR with primers TBF1-N1 and TBF1-N4. The resulting 400 bp fragment was cloned into the *Eco*RI-*Sac*I site of pRS304 (Sikorski and Hieter, 1989), and a 360 bp 9-myc epitope tag (Michaelis *et al.*, 1997) was inserted into the *Spe*I site downstream of the *TBF1* start codon. A 9-myc epitope tag was integrated at the N-terminus of the chromosomal copy of *TBF1* by transforming *Nde*I-digested pRS304-myc-TBF1 into 499UT-Y and 499UT-H. Myc-tagged Cdc13p was expressed from the centromeric plasmid pRS314-CDC13-myc9 (Taggart *et al.*, 2002). All strains were constructed in the YPH499 or YPH500 strain background (Sikorski and Hieter, 1989). The control strains were *MATa* and *MATα* versions of *TEL::URA3/VII-L* strains (Tsukamoto *et al.*, 2001), referred to here as 499UT-Y and 500UT-Y, respectively. These strains contain *URA3* adjacent to the VII-L telomere, and a 73 bp unique sequence between *URA3* and the telomere; this unique sequence is in the opposite orientation to that of the strains in Tsukamoto *et al.* (2001).

A strain expressing a *TLC1* allele in which the template region encodes the vertebrate telomere sequence instead of the yeast sequence was constructed by a two-step integration of the *tlc1h* allele from pTLC1h (Henning *et al.*, 1998) into YPH500. Correct integrants were identified by colony PCR as previously described (Henning *et al.*, 1998) and by Southern blots of *Xho*I-digested genomic DNA hybridized with an 800 bp C<sub>3</sub>TA<sub>2</sub> probe from pSP73-Sty11 (from T.de Lange) to monitor incorporation of the vertebrate sequence at the telomeres. The 500UT-H strain, carrying an entirely vertebrate telomere, was constructed by integrating the 2.4 kb *Eco*RI-*Sal*I fragment of pUT-H at the VII-L telomere of the YPH500 *tlc1h* strain (Figure 1A). The mating type of 500UT-H was switched from *MATα* to *MATa* to generate 499UT-H. Diploid strains homozygous for the *tlc1h* allele and the UT-H telomere were made by mating 499UT-H to 500UT-H. A diploid strain homozygous for the *tlc1h* mutation and heterozygous for the UT-H telomere was produced by mating 499UT-H to YPH500 *tlc1h*.

Heterozygous deletions of *EST2* were constructed by PCR amplifying *HIS3* with primers containing homology to the flanking regions of the *EST2* gene (est2HISa and est2HISb; Table I) and transforming the product into the 499UT-Y/500UT-Y and 499UT-H/500UT-H diploid strains. Heterozygous strains carrying a *rad52::LEU2* allele were made by introducing the 5 kb *Bam*HI fragment of pSM20 (Schild *et al.*, 1983) into the 499UT-Y/500UT-Y and 499UT-H/500UT-H diploid strains. Haploid spore products carrying *est2* or *rad52* mutations were identified by replica plating spore clones of dissected tetrads to medium lacking histidine or leucine, respectively.

The control disome strain for chromosome loss analysis was LS20xLS18 (Sandell and Zakian, 1993). The disome strain with vertebrate-sequence telomeres was constructed by integrating the *tlc1h* allele in LS20 (Sandell and Zakian, 1993), mating LS20 with the *kar1* strain LS15 [parent strain of LS18 with unmodified VII-L telomere (Sandell and Zakian, 1993)], and selecting on YC Can-Arg-Lys-Tyr low Ade to isolate a chromosome VII disome strain. The 2.4 kb *Eco*RI-*Sall* fragment of pUT-H was integrated at the VII-L telomere of the *LYS5 aro2 CYH2 ADE3* copy of chromosome VII (the test chromosome). The *tlc1h* disomic strain with the C<sub>3</sub>TA<sub>2</sub> telomere was grown for >100 generations before chromosome loss was measured. Chromosome loss assays were performed similarly to Sandell and Zakian (1993). Recombination was monitored by counting the number of red, cycloheximide-resistant colonies that arose.

Diploid strains were sporulated for 5–6 days at room temperature in 0.5% potassium acetate. Sporulated cells were incubated 15 min at 30°C with 0.5 mg/ml zymolyase in 1 M sorbitol and dissected on YC plates.

### Analysis of telomeres

The VII-L telomere was PCR-amplified as in Forstemann *et al.* (2000), using primers dG18-Bam (Forstemann *et al.*, 2000) and UT-Y(+) for wild-type telomeres or UT-H(-) for C<sub>3</sub>TA<sub>2</sub> telomeres (Table I). The resulting products were cloned into the pCRII-TOPO vector using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA), and sequenced using M13 primers on an Applied Biosystems 3100 automated DNA sequencer (MWG Biotech AG, Ebersberg, Germany). Telomere length was monitored by Southern blots of yeast genomic DNA digested with *Pst*I and *Xho*I and probed sequentially with the *URA3* gene, ~300 bp C<sub>1-3</sub>A from pCT300 and 800 bp C<sub>3</sub>TA<sub>2</sub> from pSP73-Sty11 (from T.de Lange). TPE was measured qualitatively by spotting 10-fold serial dilutions of overnight cultures onto YC, YC -Ura and FOA plates. Quantitative silencing assays were performed as in Gottschling *et al.* (1990).

ChIP was performed essentially as in Taggart *et al.* (2002) except that protein A and G Dynabeads were used (Dyna, Oslo, Norway). PCR products were quantitated using the Scion Image program (Scion Corporation, Frederick, MD). Polyclonal sera were used to precipitate Rap1p (Conrad *et al.*, 1990) and Rif2p (S.-C.Teng, unpublished data), while anti-Myc monoclonal antibodies (Clontech, Palo Alto, CA) were used for myc-tagged proteins. The Rif2p antiserum was raised in rabbits against full-length, glutathione *S*-transferase-tagged Rif2p purified from *Escherichia coli* and affinity purified (S.-C.Teng, unpublished data). The negative control for samples precipitated with polyclonal antibodies was a sample immunoprecipitated with polyclonal serum to the non-telomere protein Ypt1p (provided by G.Waters).

### Immunofluorescence

For immunofluorescence localization (Tham *et al.*, 2001), diploid cells were labeled using a 1:2000 dilution of affinity-purified (W.-H.Tham, unpublished data)  $\alpha$ -Rap1 polyclonal antibody (Conrad *et al.*, 1990) or haploid myc-Tbf1p cells were labeled with a 1:200 dilution of  $\alpha$ -myc monoclonal antibody (Clontech). The secondary antibodies were 1:100 dilutions of Alexa Fluor 546 and Alexa Fluor 488 (Molecular Probes, Eugene, OR).

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## Note added in proof

Using chromatin immunoprecipitation, we find that Yku80p, a subunit of the heterodimeric Ku complex, binds well to the C3TA2 telomere.