

The number of vertebrate repeats can be regulated at yeast telomeres by Rap1-independent mechanisms

Vanessa Brevet, Anne-Sophie Berthiau, Livia Civitelli¹, Pierluigi Donini¹, Vera Schramke², Vincent Géli², Fiorentina Ascenzioni¹ and Eric Gilson³

Laboratoire de Biologie Moléculaire de la Cellule, UMR5665, Centre National de la Recherche Scientifique, Ecole Normale Supérieure de Lyon, 46 Allée d'Italie, 69364 Lyon Cedex 07, ²Laboratoire d'Ingénierie et de Dynamique des Systèmes Macromoléculaires, CNRS, 31 chemin Joseph Aiguier, 13402 Marseille Cedex 20, France and ¹Istituto Pasteur-Fondazione Cenci Bolognietti, c/o Dipartimento di Biologia Cellulare e dello Sviluppo, University of Rome 'La Sapienza', via dei Sardi 70, 00185 Rome, Italy

³Corresponding author
e-mail: Eric.Gilson@ens-lyon.fr

The number of telomeric DNA repeats at chromosome ends is maintained around a mean value by a dynamic balance between elongation and shortening. In particular, proteins binding along the duplex part of telomeric DNA set the number of repeats by progressively limiting telomere growth. The paradigm of this counting mechanism is the Rap1 protein in *Saccharomyces cerevisiae*. We demonstrate here that a Rap1-independent mechanism regulates the number of yeast telomeric repeats (TG₁₋₃) and of vertebrate repeats (T₂AG₃) when *TEL1*, a yeast ortholog of the human gene encoding the ATM kinase, is inactivated. In addition, we show that a T₂AG₃-only telomere can be formed and maintained in humanized yeast cells carrying a template mutation of the gene encoding the telomerase RNA, which leads to the synthesis of vertebrate instead of yeast repeats. Genetic and biochemical evidences indicate that this telomere is regulated in a Rap1-independent manner, both in *TEL1* and in *tel1Δ* humanized yeast cells. Altogether, these findings shed light on multiple repeat-counting mechanisms, which may share critical features between lower and higher eukaryotes.

Keywords: evolution/TEL1/telomerase/telomere/yeast

Introduction

Telomeres are nucleoprotein structures found at the ends of all eukaryotic linear chromosomes (reviewed in Blackburn and Greider, 1995; Zakian, 1995). They play essential roles in the maintenance of genome integrity and in the control of cell proliferation. They specifically cap the ends of chromosomes such that they are not recognized by DNA damage checkpoints and repair systems (Sandell and Zakian, 1993). They are also involved in the control of gene expression and in the spatial and functional organization of chromosomes (Gottschling *et al.*, 1990; reviewed in Gilson *et al.*, 1993).

In most eukaryotes, the sequence of telomeric DNA consists of a tandem array of short guanine-rich repeats. It is an oriented structure with the G-rich strand running 5' to 3' towards the distal end of the chromosome and ending as a short single-stranded 3' overhang. Telomeric DNA decreases in length as cells divide. This is due to a replication defect in the most distal telomeric sequence, which cannot be achieved by the standard polymerase machinery, and also to nuclease activities coupled to replication (reviewed in Lingner *et al.*, 1995). The dynamics of telomere length control cell proliferation and genome integrity, triggering senescence, apoptosis and chromosomal instability (reviewed in Blackburn, 2001). In many organisms, telomere shortening is compensated by telomerase, a unique reverse transcriptase, which specifically extends the 3' G-rich telomeric strand (reviewed in Blackburn, 2000). This enzyme appears to be constitutively expressed in many unicellular organisms, including the budding and fission yeasts, and to be highly regulated during the development of metazoans.

Usually, the telomere length of telomerase-positive cells is maintained at a constant mean value (reviewed in McEachern *et al.*, 2000). Remarkably, the mean telomere length exhibits variations between yeast strains or human individuals. This cellular determination of the mean number of telomeric repeats is best represented by the so-called counting mechanism, that is the ability of the cell to retain the 'memory' of the number of telomeric repeats corresponding to a constant mean value. This mechanism is involved in the progressive return to equilibrium of too short telomeres either during the *de novo* formation of telomeres (Szostak and Blackburn, 1982; Barnett *et al.*, 1993) or when a single telomere is abruptly shortened (Marcand *et al.*, 1999). Another demonstration of repeat counting is the ability of telomeric repeats or telomeric protein-binding sites inserted at an internal position from the telomere, even when misorientated, to be taken as part of the total telomere length (Marcand *et al.*, 1997; Ray and Runge, 1999b; Grossi *et al.*, 2001). Consequently, the number of telomeric repeats distal to this internal sequence decreases. In budding yeast, the counting mechanism appears to be based on a progressive decline in the rate of telomere elongation when the number of repeats increases until elongation is balanced by degradation (Marcand *et al.*, 1999).

In budding yeast, an important counting factor is Rap1. An excess of Rap1 bound to a telomere causes repression of telomerase activity *in cis*, thereby creating a negative feedback loop that contributes to the setting of telomere length (Kyrion *et al.*, 1992; Krauskopf and Blackburn, 1996; Marcand *et al.*, 1997, 1999; Ray and Runge, 1999b). Two factors interacting with Rap1, Rif1 and Rif2, are also involved in this Rap1-dependent regulation (Wotton and Shore, 1997). There is now increasing evidence that

mechanisms involving protein binding to telomeric DNA sequences and limiting telomere elongation are conserved in distantly related organisms. For instance, in fission yeast, a deletion of *TAZI*, a gene encoding a telomeric DNA-binding protein, dramatically increases telomere length (Cooper *et al.*, 1997). Interestingly, although the fission yeast SpRap1 negatively regulates telomere elongation like its budding yeast ortholog, it is not directly bound to telomeric DNA, but associates to the telomere through an interaction with Taz1 (Chikashige and Hiraoka, 2001; Kanoh and Ishikawa, 2001; Park *et al.*, 2002). In human cells, the known telomeric DNA-binding proteins are TRF1 and TRF2 (Chong *et al.*, 1995; Bilaud *et al.*, 1996, 1997; Broccoli *et al.*, 1997). Reminiscent of the properties of the yeast Rap1, an excess of TRF1 and TRF2 inhibits telomere elongation (van Steensel and de Lange, 1997; Smogorzewska *et al.*, 2000). Moreover, their targeting to a single telomere leads to telomere shortening, indicating that they act in *cis* to limit telomere elongation (Ancelin *et al.*, 2002). As in fission yeast, the human ortholog of Rap1 does not bind to telomeric DNA, but is likely to be targeted to telomeres via its interaction with TRF2 (Li *et al.*, 2000). Interestingly, TRF2 can shorten telomeres in the absence of telomerase (Ancelin *et al.*, 2002; Karlseder *et al.*, 2002).

A fascinating connection between the counting mechanism of telomere length and chromosome end stability has been revealed by the functions of the PI3-like kinase ATM, which plays an important role in the checkpoint of double-strand break in human cells, and of its ortholog in budding yeast *TEL1* (reviewed in Pandita, 2002). Loss of function of these proteins leads to a new steady-state number of telomeric repeats, shorter than the wild type. In *tel1Δ* yeast cells, in contrast to wild-type cells, non-telomeric Rap1-binding sites are not counted (Ray and Runge, 1999a). Moreover, in the absence of *TEL1*, a deletion of the C-terminal part of Rap1 as well as the disruption of *RIF1* or *RIF2* have no effect on mean telomere length (Craven and Petes, 1999; Ray and Runge, 1999a). This led us to investigate Rap1-independent mechanisms of telomere length regulation in budding yeast.

Results

Yeast repeats are counted both in *TEL1* and in *tel1Δ* cells while the vertebrate repeats are counted only in *tel1Δ* cells

In order to characterize the counting factor acting in *tel1Δ* cells, we asked whether internal stretches of the budding yeast TG_{1-3} repeats or of the vertebrate T_2AG_3 repeats could be taken into account for telomere length regulation in the absence of *TEL1*. To this end, the left extremity of chromosome VII beyond the *ADH4* gene was replaced by a newly formed *URA3*-tagged telomere containing the sequences $(TG_{1-3})_{23}$ or $(T_2AG_3)_{10}$ inserted at the vicinity of the distal TG_{1-3} repeats. We also inserted in the same location a sequence containing four non-telomeric Rap1-binding sites [named $(RAP1)_4$] and a portion of the *RAD52* gene (named NR). The counting effect of the inserted sequences was evaluated by measuring the size of a terminal restriction fragment containing part of the *URA3* gene (Marcand *et al.*, 1997; Ray and Runge, 1999b; Figure 1).

When the control sequence NR is inserted close to the telomere, the length of the distal TG_{1-3} repeats, is identical to the mean length of the strains without insert, i.e. ~300 nucleotides (nt) for *TEL1* and ~160 nt for *tel1Δ* cells (Figure 1B, lanes d and h, and C; data not shown). This validates the use of the NR sequence as a non-counted control. As expected, in *TEL1* cells, both $(TG_{1-3})_{23}$ and $(RAP1)_4$ shorten the distal repeats as compared with NR, indicating that they are taken into account for length regulation (Figure 1B, lanes a and c, and C). In *tel1Δ* cells, the shortening was more pronounced for $(TG_{1-3})_{23}$ than for $(RAP1)_4$ (~45 nt versus 20 nt) (Figure 1B, lanes e and g, and C). Thus, the yeast telomeric repeats mediate a more potent counting mechanism than non-telomeric Rap1 sites when *TEL1* is inactivated. The counting effect of yeast repeats was confirmed in another *tel1Δ* background using an internal stretch of 270 nt of TG_{1-3} repeats (see Supplementary figure 1A, available at *The EMBO Journal* Online). In this case, the internal stretch of TG_{1-3} repeats is flanked on both sides by a FRT site, which can be excised upon induction of the expression of the *FLP1* gene in the presence of galactose. Using this experimental design, we demonstrated previously that *in vivo* telomerase activity is gradually repressed upon telomere elongation, suggesting that the accumulation of counting factors leads to a progressive inhibition of telomerase activity (Marcand *et al.*, 1999). As in wild-type cells, we observed a progressive decline in the elongation rate as a function of telomere length in *tel1Δ* cells, suggesting a seemingly similar type of counting mechanism in *TEL1* and in *tel1Δ* yeast cells (see Supplementary figure 1B and C).

Strikingly, the $(T_2AG_3)_{10}$ sequence is correctly counted in *tel1Δ* cells, leading to a shortening of 49 nt as compared with NR (Figure 1B, lane f, and C). Importantly, the $(T_2AG_3)_{10}$ insert is not taken into consideration for telomere length regulation in the presence of *TEL1* (Figure 1B, lane b, and C). We conclude that a cryptic Rap1-independent counting mechanism, which is revealed in the absence of *TEL1*, can accommodate both T_2AG_3 and TG_{1-3} repeats, while the Rap1-dependent mechanism is specific for TG_{1-3} repeats.

Progressive replacement of TG_{1-3} by T_2AG_3 repeats in humanized yeast cells

The ability of *tel1Δ* cells to count subtelomeric T_2AG_3 repeats (see above) prompted us to investigate whether the number of T_2AG_3 repeats can be regulated over the entire length of a yeast telomere. To this end, we made use of a humanized yeast strain, which carries the *tlc1-human* (*tlc1-h*) allele of the RNA telomerase *TLC1* gene. The presence of the *tlc1-h* allele results in the incorporation of T_2AG_3 instead of TG_{1-3} repeats at the end of yeast chromosomes (Henning *et al.*, 1998). Shortly after the growth of haploid spores carrying *tlc1-h* instead of *TLC1*, the mean length of the Y' telomeres appears stable but shorter than telomeres of cells originating from wild-type *TLC1* spores (Henning *et al.*, 1998). We repeated this experiment starting from the heterozygote *TLC1/tlc1-h* (designated C6.1 in Henning *et al.*, 1998). We measured the Y' telomere length in several *tlc1-h* haploid cells after ~50 generations post-germination and confirmed that telomeres were shortened of ~100–150 nt as compared

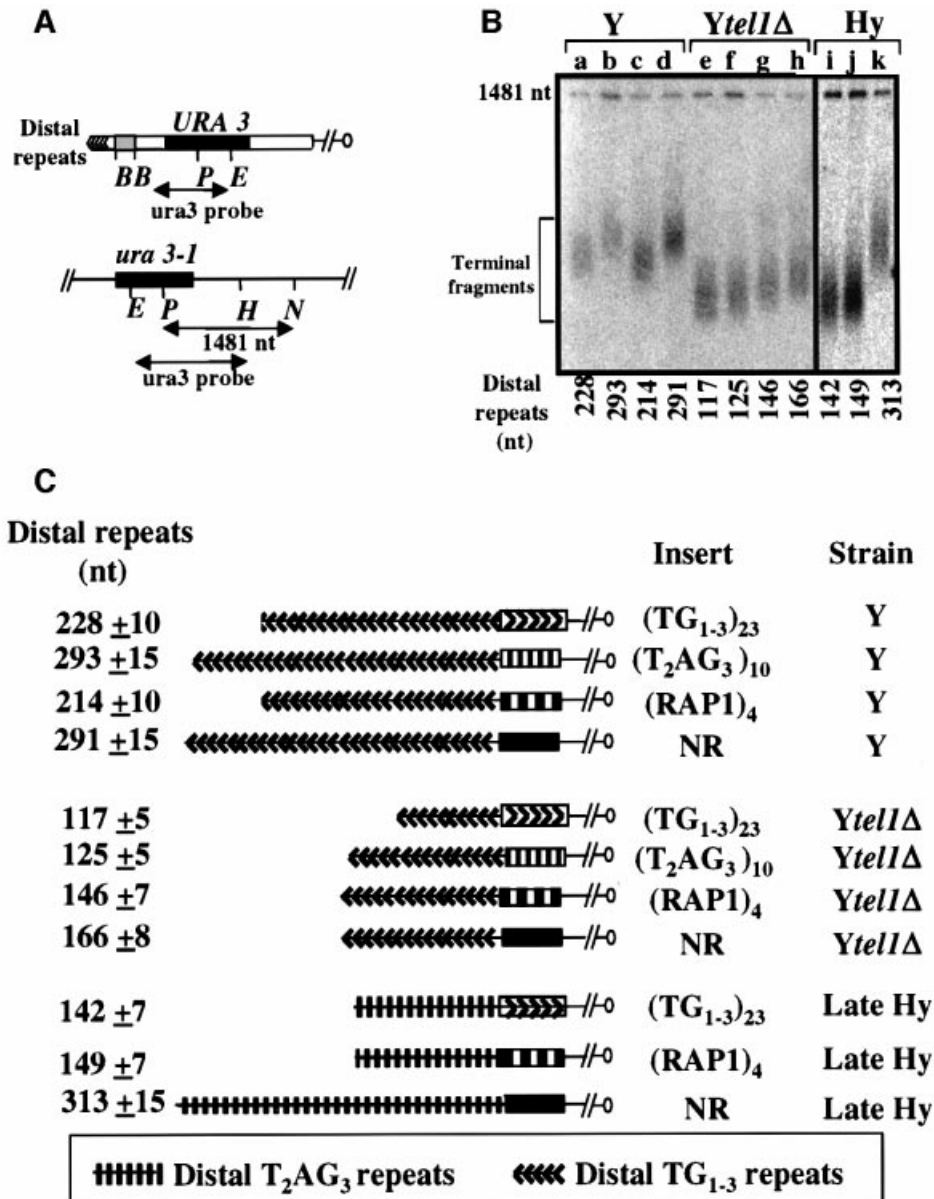


Fig. 1. In *tellΔ* cells TG₁₋₃ and T₂AG₃ repeats are counted by Rap1-independent pathways. (A) Schematic representation of the modified VII-L telomere used to determine the counting capacity of the internal sequence. The positions of the *EcoRV* (E), *SapI* (P), *NaeI* (N), *HindIII* (H) and *BamHI* (B) restriction sites are indicated. The gray box bracketed by *BamHI* sites indicates the position of the inserted sequences (see Materials and methods). (B) Genomic DNA of the Y, *YtellΔ* and Hy strains was digested with *SapI* and *NaeI* and hybridized with the *ura3* probe. The median length of the telomeric restriction fragment was calculated using a set of molecular weight markers (not shown) and the non-telomeric 1481 nt fragment as an internal control. In order to obtain the length of the distal telomeric repeats, 378 nt was subtracted from the size of the measured terminal fragment, as shown below each lane. The inserted sequence is (TG₁₋₃)₂₃ in lanes a, e and i; (T₂AG₃)₁₀ in lanes b and f; (RAP1)₄ in lanes c, g and j; and NR in lanes d, h and k. (C) Schematic representation of the results.

with *TLC1* cells (Figure 2A, B, lanes b, e and h, and C, lane b; data not shown). One *TLC1* haploid and one *tlc1-h* haploid were chosen for further analysis, and were named Y and Hy cells, respectively. After ~500 generations of Hy cells, we noticed a decrease in the intensity of the Y' telomere hybridization signal with a TG₁₋₃ probe (Figure 2B, lanes e and f) as compared with a T₂AG₃ probe or with a Y' probe (Figure 2B, lanes b and c, and h and i, respectively). Remarkably, the long-term growth of Hy cells also led to a slight increase in the length of the Y' telomeres (Figure 2B, lane i, and D, lanes e and l). This

elongation stopped after ~500 generations at a new mean length, which is still shorter than wild-type yeast telomeres. This new length setting appears to be stable over an extended period of growth, up to 800 generations after germination (data not shown). It does not seem to result from a selection of cells that grow faster since a slight increase in generation time is observed for late cultures of Hy cells and since it occurs both when cells are serially diluted from liquid culture or upon successive restreaking on agar plates (data not shown). Because of these observations, we will refer hereafter to early Hy cells

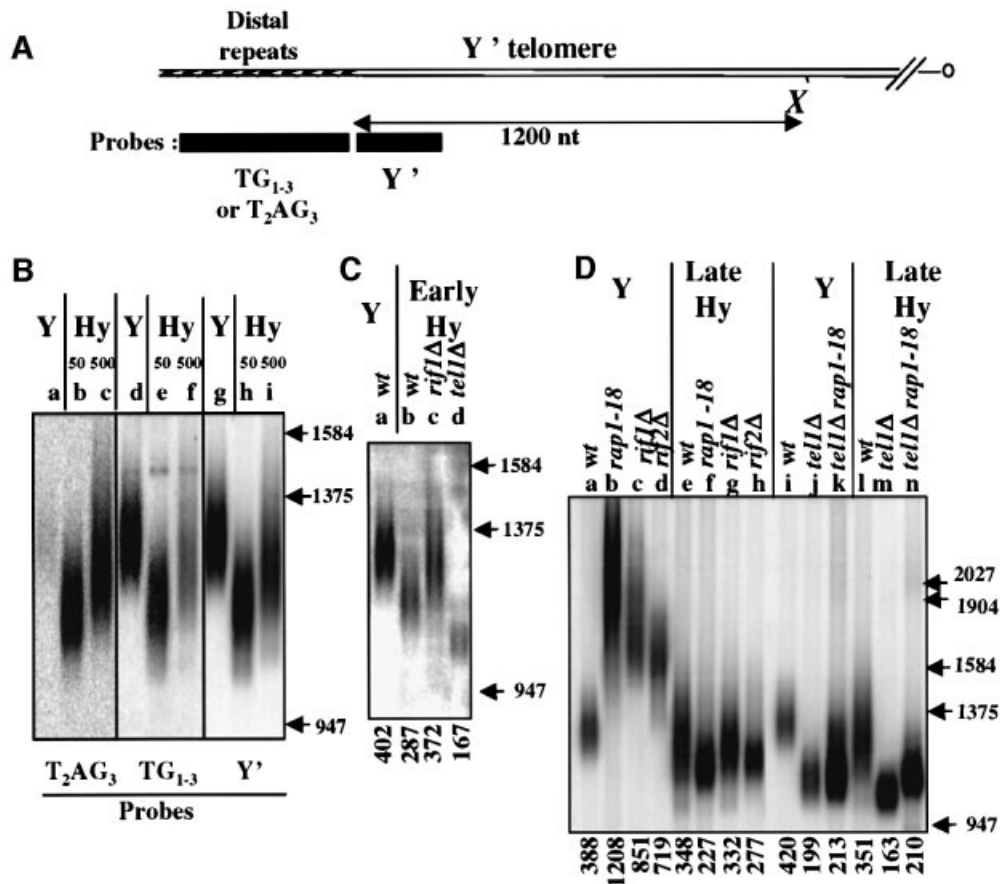


Fig. 2. Maintenance of Y' telomere in humanized yeast. (A) Schematic representation of yeast telomeres associated with a Y' subtelomeric element, the position of the invariable *XhoI* (X) site is reported. Digestion of genomic DNA by *XhoI* allows to measure the size of the terminal fragment of all Y' telomeres in the cells. The different probes used for Southern blot analysis are shown. The length of the distal repeats was estimated by subtracting 1200 nt from the measured length of the terminal *XhoI* fragment. (B) Example of Y' telomere Southern blot hybridized successively to a T₂AG₃, TG₁₋₃ or Y' probe. The number of generation after germination is indicated for the Hy cells. Lanes a, d and g: Y cells; lanes b, e and h: Hy cells after 50 generations (early passages); lanes c, f and i: Hy cells after 500 generations (late passages). (C and D) The *XhoI* blots were hybridized with the Y' probe. wt: wild type for the *RAP1*, *RIF1*, *RIF2* and *TEL1* genes. In (B–D), the molecular weight markers, used to measure the mean telomere lengths, are indicated in nt. In (C) and (D), the length of distal repeats is indicated at the bottom of each lane.

as those that had grown for <100 generations after germination, and to late Hy cells as those that had grown for >300 generations.

Formation and maintenance of a telomere made exclusively of T₂AG₃ repeats

In order to investigate whether a telomere made solely of T₂AG₃ repeats can be maintained in Hy cells, the left extremity of chromosome VII was replaced in Y and Hy cells by an *URA3*-containing DNA ending with a stretch of 10 T₂AG₃ repeats. In Y cells, such a construct led to the addition of TG₁₋₃ repeats distally to the T₂AG₃ repeats (data not shown). This is in agreement with previous work showing that various types of telomeric repeats can be used as a seeding sequence (Szostak and Blackburn, 1982; Lustig, 1992). In late Hy cells, the majority of randomly selected *URA3*⁺ clones exhibited a restriction pattern indicative of a successful seeding event, i.e. a smeary signal for the VII-L terminal restriction fragment probed with an *ura3* probe (Figure 3A and B, lanes g and k; data not shown).

The *URA3*-tagged telomeric DNA fragment was PCR-amplified from the genomic DNA of one of these clones,

and the recovered plasmids contained an insert in the size range of 400–650 nt that hybridizes to a T₂AG₃ probe (data not shown). The sequencing of four randomly chosen plasmid DNAs shows the presence of an uninterrupted array of T₂AG₃ repeats over the entire length of the cloned telomere, demonstrating the *de novo* formation of a telomere composed solely of T₂AG₃ repeats. This T₂AG₃-only telomere can be formed and propagated normally when the *RAD52* gene is disrupted (data not shown). Thus, the T₂AG₃-only telomere is unlikely to be maintained by homologous recombination. We have been unable to disrupt the *EST2* gene in Hy cells (data not shown). Based on the *RAD52*-independence and the failure to introduce an *EST2* disruption, we conclude that telomerase plays a critical role in the maintenance of the T₂AG₃-only telomere.

The length profile of the T₂AG₃-only telomere exhibits an increased heterogeneity as compared with a *URA3*-tagged telomere in Y cells (Figure 3B, compare lane a with g and k). During 40 restreaks that correspond to ~800 generations, the length and heterogeneity of the T₂AG₃-only telomere appears relatively well preserved, although some fluctuations can be observed, with a mean

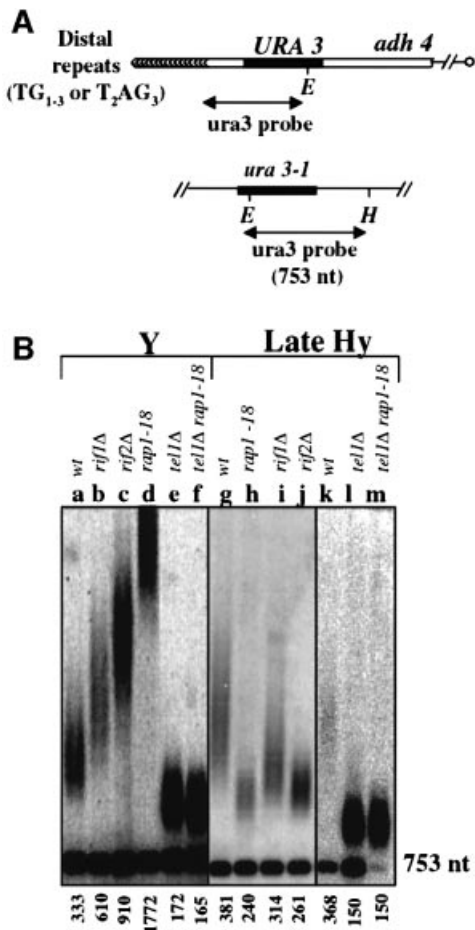


Fig. 3. Maintenance of the T₂AG₃-only telomere. (A) Schematic representation of the modified VII-L telomere used to monitor the length of the wild type and of T₂AG₃-only telomere. A representation of the *ura3-1* allele is shown to indicate the existence of a 753 nt fragment hybridizing with the *ura3* probe. (B) Genomic DNA was digested with *EcoRV* and *HindIII* and hybridized with the *ura3* probe. The median length of the telomeric restriction fragment was calculated using a set of molecular weight markers (not shown) and using the *ura3-1* 753 nt fragment as an internal control. 720 bp was subtracted from this value to give the size of the distal TG₁₋₃ repeats (Y strains) or the distal T₂AG₃ repeats (Hy strains). wt: wild type for the *RAP1*, *RIF1*, *RIF2* and *TEL1* genes.

length ranging from 350 to 500 nt (data not shown). We conclude that the length of the T₂AG₃-only telomere can be regulated in budding yeast, although the mechanism involved appears less efficient than the one acting in *TLC1* cells.

The Rap1 pathway does not limit elongation of telomeres made principally or exclusively of T₂AG₃ repeats

We then investigated genetically whether the Rap1 pathway plays a role in telomere length regulation in Hy cells. To this end, we introduced in *tlc1-h* cells carrying an *URA3*-tagged telomere the *rif1Δ*, *rif2Δ* and *rap1-18* mutations, which overelongate telomeres in *TLC1* cells by disrupting the Rap1-mediated *cis*-repression of telomerase activity (Kyrion *et al.*, 1992; Wotton and Shore, 1997) and the *tel1Δ* mutation, which shortens telomeres by inhibiting telomerase activity (Craven and Petes, 1999).

Since the sporulation efficiency of *tlc1-h/tlc1-h* homozygote was very poor, probably due to their telomeric defects, the production of mutated haploid cells from the diploids was very inefficient. Consequently, the mutations were introduced by gene replacement in haploid cells of the Y and Hy strains. Several independent transformants were analyzed and in each case the telomeric phenotypes were similar. In addition, each mutant was backcrossed to *tlc1-h* cells of opposite mating type and was shown to be complemented by the wild-type allele (data not shown). For the sake of clarity, we will present in Figures 2 and 3 the results obtained for a single transformant of each construction.

As expected, the Y' telomeres and the *URA3*-tagged telomere were overelongated in Y cells deleted for *RIF1* (Figures 2D, lane c, and 3B, lane b). When the *RIF1* disruption is introduced into early Hy cells, this also leads to overelongation of Y' telomeres as compared with *RIF1* Hy cells (Figure 2C, lane c). However, and strikingly, when the same *RIF1* disruption was introduced into late Hy cells, a faint shortening of Y' telomeres was observed (Figure 2D, lane g). Similarly, the *rap1-18* allele or the deletion of *RIF2* leads to Y' telomere shortening in late Hy cells (Figure 2D, lanes f and h). The *rap1-18*, *rif1Δ* and *rif2Δ* mutations were also found to decrease the length of the T₂AG₃-only telomere in late Hy cells (Figure 3B, lanes h–j). In contrast to what has been reported (Wotton and Shore, 1997), the *RIF1* disruption has a less pronounced deregulation effect than the *RIF2* disruption at the *URA3*-tagged telomere of Y cells (Figure 3B, lanes b and c). The reason for this unexpected hierarchy between the mutation phenotypes is unknown, but might be related to strain differences. It is noteworthy that the relative strength between *rif1Δ* and *rif2Δ* is opposite at the Y' telomeres (Figure 2D, lanes c and d).

The fact that the length of Y' telomere slightly increases in early but not in late *rif1Δ* Hy cells raises the possibility that the repressive effect of the Rap1–Rif complex is lost in late Hy cells because of the progressive replacement of TG₁₋₃ repeats by T₂AG₃ (see above and Figure 2B). This hypothesis predicts that TG₁₋₃ repeats in late Hy cells are still able to negatively regulate telomere length. This was directly tested by forming in late Hy cells composite telomeres containing an internal (TG₁₋₃)₂₃ or (RAP1)₄ sequence and distal T₂AG₃ repeats. The lengths of these telomeres are maintained at a mean value, which is twice as short as one of the T₂AG₃-only telomere (Figure 1B, lanes i and j, and C). This reveals a very potent counting effect in late Hy cells exerted by the internal TG₁₋₃ repeats and the non-telomeric Rap1 sites. This counting is even stronger than the one observed with an identical amount of internal repeats in Y cells (Figure 1C). This could be explained by a greater sensitivity of the humanized telomerase and/or of the T₂AG₃-enriched telomeres to Rap1-mediated repression.

In *TLC1* cells, the absence of *TEL1* revealed a cryptic length regulation pathway recognizing internal T₂AG₃ repeats (see above and Figure 1). Therefore, the *TEL1* gene product might be inoperative at the telomeres of Hy cells, explaining the ability of these cells to regulate telomere length. However, in Hy cells, the deletion of *TEL1* leads to a pronounced shortening of the Y' telomere (Figure 2C, lane d, and D, lane m) and of the T₂AG₃-only telomere

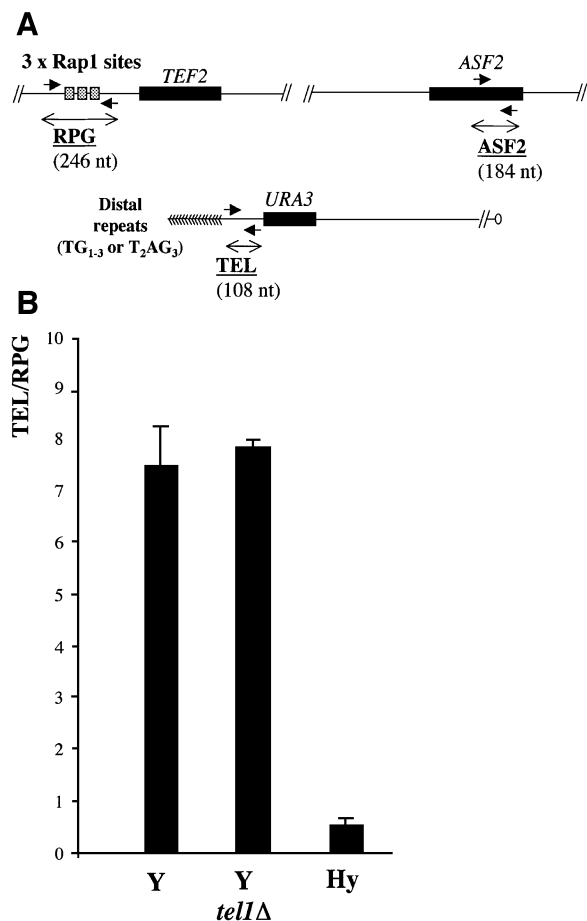


Fig. 4. The binding of Rap1 to the T_2AG_3 -only telomere is impaired. A myc tag epitope was inserted at the C-terminus of the *RAP1* endogenous gene in Y, Y *tel1Δ* and Hy strains carrying an *URA3*-tagged telomere. (A) Schematic representation of the three DNA fragments analyzed for Rap1 binding by ChIP and by real-time PCR. (B) Results of the immunoprecipitation are expressed as the ratio of the immunoprecipitated TEL DNA to that of the RPG DNA. The average value for at least three experiments is given in the diagram as well as the estimated standard deviation.

(Figure 3B, lane l). These data indicate that *TELI* is required for a proper length regulation in Hy cells. The double mutation *tel1Δ rap1-18* does not lead to an additional shortening of the T_2AG_3 -only telomere in late Hy cells (Figure 3B, lane m), suggesting that a Rap1-dependent elongation in Hy cells also requires *TELI* activity. We believe that the very short Y' telomeres in Hy *tel1Δ* cells (Figure 2D, lane m) might be attributed to the potent 'counting capacities' of TG_{1-3} repeats in Hy cells (Figure 1C).

The T_2AG_3 -only telomeric DNA appears devoid of Rap1

In order to assay the binding of Rap1 to the T_2AG_3 -only telomere in Hy cells, we performed chromatin immunoprecipitation experiments (ChIP) with formaldehyde-mediated cross-linked cells expressing a myc-tagged version of Rap1. The *RAP1-MYC* Hy cells behave as *RAP1* Hy cells with respect to viability, growth rate and telomere length (data not shown). However, we noticed that the telomeres of *RAP1-MYC* Y cells were slightly

longer than those of *RAP1* cells (+30 nt). This, together with the absence of telomere length change due to Rap1-myc expression in Y *tel1Δ* cells, suggests that the addition of the myc tag to the C-terminus of Rap1 induces a minor loss of function of the C-terminus of Rap1. This is not expected to affect the binding of Rap1 to its natural targets, both because the cells expressing Rap1-myc do not show any growth defect and because the DNA-binding properties of Rap1 depend mainly on the central domain. Overall, we considered that the Rap1-myc fusion protein is largely functional.

The DNA recovered from the immunoprecipitates was accurately quantified by real-time PCR (Ladenburger *et al.*, 2002). The RPG PCR fragment of the *TEF2* promoter, which is bound *in vivo* by three Rap1 proteins simultaneously (De Sanctis *et al.*, 2002), was used as a positive non-telomeric control for Rap1 binding (Figure 4A). We used as a negative control the *ASF2* open reading frame, which is known to be devoid of Rap1 sites (Liu *et al.*, 2002) (Figure 4A). The specificity of our ChIP procedure was estimated by the ratio of the immunoprecipitated RPG to *ASF2* DNA. This ratio fluctuated between 8 and 12 in all the experiments performed with Y, Y *tel1Δ* and Hy cells (data not shown). When the same ChIP experiments were performed but with cells expressing the wild-type Rap1 protein or in the absence of formaldehyde cross-linking, we were unable to detect any specific amplification of the RPG fragment. We conclude that our ChIP experiments were reliable since our conditions led to an expected specific enrichment in Rap1-bound DNA at the *TEF2* promoter in all types of yeast cells used in this study.

The TEL PCR fragment lies adjacent to an *URA3*-tagged telomere (Figure 4A). It does not contain a Rap1-binding site, but its immediate juxtaposition to the telomeric DNA guarantees its preferential coprecipitation with telomeric DNA. In order to compare telomeric versus non-telomeric binding of Rap1, we measured the ratio between the quantities of TEL to RPG DNA in the immunoprecipitates (TEL/RPG in Figure 4B). As expected, we found a 7.5-fold enrichment of TEL DNA as compared with RPG DNA in ChIP with Y cells. This value could be a slight underestimation of the amount of telomeric Rap1 since the TEL fragment does not overlap with the telomeric repeats (see above). In any case, the enrichment is compatible with the number of Rap1 proteins bound at a wild-type yeast telomere, which was estimated to be ~18 (Gilson *et al.*, 1993). Remarkably, there is no enrichment of TEL over RPG in experiments performed with Hy cells (Figure 4B). To the contrary, less TEL than RPG DNA was immunoprecipitated, leading to a TEL/RPG ratio of 0.3. Since RPG is expected to be bound by three Rap1 molecules, one can infer that fewer than three Rap1 molecules are bound to the TEL fragment in Hy cells. This absence or quasi-absence of Rap1 from the T_2AG_3 -only telomeric DNA cannot be explained by telomere shortening, since the mean length of the *URA3*-tagged telomere in Y and Hy cells are roughly similar (Figure 3B, lanes a, g and k). Moreover, ChIP experiments performed with Y *tel1Δ* cells that exhibit a mean length much shorter than wild type (Figure 3B, lane e) leads to an enrichment of TEL over RPG similar to that observed in Y cells. The recovery of a seemingly equal amount of TEL DNA from Y and Y *tel1Δ* cells could

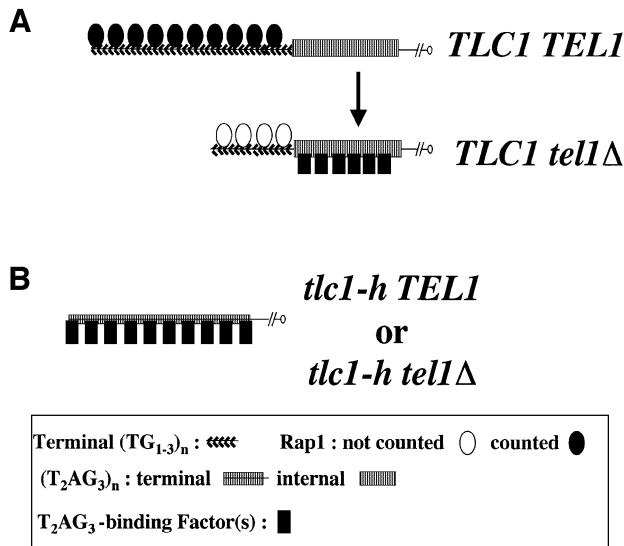


Fig. 5. Multiple pathways regulate the number of vertebrate telomeric repeats at chromosome ends of budding yeast. The symbols are explained at the bottom of the figure. **(A)** In *tel1Δ* cells, the Rap1-counting pathway is no more operative although Rap1 stills binds to telomeres, as shown by ChIP assays. Our data indicate that unknown counting factors able to recognize T_2AG_3 repeats set the mean telomere length in *tel1Δ* cells. Whether the T_2AG_3 -binding factor is associated to vertebrate repeats in *TEL1* cells is unknown. **(B)** In humanized yeast, a T_2AG_3 -only telomere can be formed and maintained at a regulated mean length. Noteworthy, this regulation can occur both in *TEL1* and in *tel1Δ* cells and can co-habit with a Rap1-dependent regulation pathway when TG_{1-3} repeats or Rap1 sites are inserted internally. Whether the T_2AG_3 -binding factor(s) involved in length regulation is (are) the same in *TLC1 tel1Δ* and in *tlc1-h* cells is not known.

be explained if the Rap1 molecules in the cross-linked telomeric chromatin are more exposed to antibodies in *tel1Δ* cells and/or if the density of telomeric and subtelomeric Rap1 molecules is higher in *tel1Δ* than in wild-type cells. Nevertheless, these data clearly show that in Y cells, a large number of Rap1 molecules are bound to telomeres in the absence of *TEL1*. In conclusion, the telomerase-repressive functions of Rap1 appear inoperative at telomeres made principally or exclusively of T_2AG_3 repeats because of the absence or quasi-absence of Rap1 associated *in vivo* to vertebrate repeats.

Discussion

Rap1-independent counting of vertebrate repeats in budding yeast

The major finding of this work is to have revealed Rap1-independent mechanisms capable of counting the number of yeast telomeric repeats (TG_{1-3}) and of vertebrate telomeric repeats (T_2AG_3) at the telomeres of budding yeast. This is based upon two distinct experimental situations (depicted in Figure 5). In the absence of *TEL1*, vertebrate repeats inserted at the internal side of a single telomere are taken into account for telomere length regulation (Figure 5A). In an identical setting, authentic yeast repeats (TG_{1-3}) are also counted in a seemingly Rap1-independent manner (Craven and Petes, 1999; Ray and Runge, 1999a; this work). In *tlc1-h* cells, also known as humanized yeast (Henning *et al.*, 1998), a T_2AG_3 -only telomere formed only by vertebrate repeats can be

maintained at a regulated mean length (Figure 5B). In agreement with these data, Alexander and Zakian (2003) also show that a fully vertebrate telomere is maintained at a regulated telomere length (Alexander and Zakian, 2003). The disruption of *TEL1* in humanized yeast leads to shorter but stable telomeres, like in wild-type telomerase yeast. Thus, while the presence of *TEL1* does not allow the counting of internal vertebrate repeats in a wild-type telomerase context, the mean length of a T_2AG_3 -only telomere appears to be regulated both in *TEL1* and in *tel1Δ* humanized yeast. Overall, these differential effects of *TEL1* between *TLC1* and *tlc1-h* yeast on the regulation of vertebrate repeats raise the interesting possibility of multiple, possibly overlapping, mechanisms able to regulate the number vertebrate repeats at budding yeast telomeres.

Telomere length regulation in *tel1Δ* cells

The cryptic pathway(s) revealed in *tel1Δ* cells does (do) not seem to be active in *TEL1* cells, since the deletion of the C-terminus of Rap1 in the presence of *TEL1* leads to a fully deregulated telomere (Kyrion *et al.*, 1992). One explanation would be that *TEL1* has a dual effect, being both required for the yeast repeat-specific pathway and suppressing the cryptic pathway(s).

What is the nature of the factors able to regulate telomere length and to recognize yeast and vertebrate repeats in *tel1Δ* cells? Our data and those of others (Craven and Petes, 1999; Ray and Runge, 1999a) clearly eliminate Rap1, and to our knowledge there are no known *Saccharomyces cerevisiae* factors able to specifically bind both types of repeats. Alternatively, yeast and vertebrate repeats might be bound by distinct counting factors. The product of the *TBF1* gene might be such a candidate because it binds T_2AG_3 repeats with high affinity, both *in vitro* (Liu and Tye, 1991; Brigati *et al.*, 1993) and *in vivo* (Koering *et al.*, 2000; Alexander and Zakian, 2003).

Humanized yeast as a model to study telomere functions

Long-term cultures of humanized yeast resulted in a change in telomere length regulation, switching from a partially Rap1-dependent mode with short telomeres in early passages to a Rap1-independent mode with longer telomeres in late cultures. Although less pronounced, the elongation of telomeres in late *tlc1-h* cells is reminiscent of the runaway phenotype described previously for certain template mutations of the *Kluyveromyces lactis* telomerase (McEachern and Blackburn, 1995). This is likely to be explained by a progressive replacement of TG_{1-3} by T_2AG_3 repeats, in agreement with the existence of a turnover mechanism that continuously degrades and resynthesizes telomeric repeats (Krauskopf and Blackburn, 1998). In agreement with this hypothesis, we show that an internal stretch of 23 TG_{1-3} repeats or an array of non-telomeric Rap1 sites can play a role in length regulation of telomeres in late humanized yeast. This provides an explanation of the lengthening of Y' telomeres in late Hy cells that correlates with a progressive decline in the number of residual yeast repeats.

digested with *EcoRI* and amplified with hyb-anchor and pTUF primers. The PCR product was controlled by gel electrophoresis, which revealed the characteristic smear of telomeric fragments. Subsequently, after purification, it was cloned into the pGEMT vector (Promega) and sequenced.

ChIP and quantitative PCR using the LightCycler instrument (Roche Biochemicals) were performed as described previously (Strahl-Bolsinger *et al.*, 1997; Schramke *et al.*, 2001). We used three pairs of primers: primers in the *ASF2* gene as a negative control (ORF, primers: GCGCTCTTTCTACTCTTAATCC, TCAGGTTGGCCCTTATATGTGGTG); primers surrounding three Rap1-binding sites inside the promoter of *TEF2* (RPG, primers: GCCGGCTGTAGGGGGCCG-CGCAT, CTTTCCAGCATAGTCGAAGAGA); and primers located between the telomere and the subtelomeric *URA3* gene (TEL, primers: GCAATATTTGAGAAGATGCGCCAGC, TAATAACTGATATAA-TTAAATTGAA). The quantitative analyses by real-time PCR were performed mainly as described in Ladenburger *et al.* (2002). Briefly, genomic DNA of known concentration was amplified by using the relevant primers to produce a standard curve. Identical PCRs were performed with the DNA from immunoprecipitated chromatin as a template. The concentration of a given DNA fragment (either RPG, TEL or *ASF2*) was determined by extrapolation to the relevant standard curve.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Acknowledgements

We would like to thank P.P.Liu, D.Gottschling and D.Shore for various strains, plasmids and advice. We would like also to thank M.Brunori for critical reading of the manuscript and M.K.Alexander and V.A.Zakian for communicating unpublished results. The work in E.G.'s laboratory was supported by 'La Ligue Nationale contre le Cancer'. Work in the laboratory of V.G. was supported by the 'Association pour la Recherche contre le Cancer' and by the 'Fondation pour la Recherche Medicale'. Work in F.A.'s laboratory was supported by University of Rome 'La Sapienza' (La cellula di lievito come modello in biologia cellulare e per lo sviluppo di sistemi biotecnologici).

References

- Alexander,M.K. and Zakian,V.A. (2003) Rap1p telomere association is not required for mitotic stability of a C₃TA₂ telomere in yeast. *EMBO J.*, **22**, 1688–1696.
- Ancelin,K., Brunori,M., Bauwens,S., Koering,C.E., Brun,C., Ricoul,M., Pommier,J.P., Sabatier,L. and Gilson,E. (2002) Targeting assay to study the *cis* functions of human telomeric proteins: evidence for inhibition of telomerase by TRF1 and for activation of telomere degradation by TRF2. *Mol. Cell Biol.*, **22**, 3474–3487.
- Barnett,M.A., Buckle,V.J., Evans,E.P., Porter,A.C.G., Rout,D., Smith,A.G. and Brown,W.R.A. (1993) Telomere directed fragmentation of mammalian chromosomes. *Nucleic Acids Res.*, **21**, 27–36.
- Bianchi,A. and de Lange,T. (1999) Ku binds telomeric DNA *in vitro*. *J. Biol. Chem.*, **274**, 21223–21227.
- Bilaud,T., Koering,C.E., Binet-Brasselet,E., Ancelin,K., Pollice,A., Gasser,S.M. and Gilson,E. (1996) The telobox, a Myb-related telomeric DNA binding motif found in proteins from yeast, plants and human. *Nucleic Acids Res.*, **24**, 1294–1303.
- Bilaud,T., Brun,C., Ancelin,K., Koering,C.E., Laroche,T. and Gilson,E. (1997) Telomeric localization of TRF2, a novel human telobox protein. *Nat. Genet.*, **17**, 236–239.
- Blackburn,E.H. (2000) The end of the (DNA) line. *Nat. Struct. Biol.*, **7**, 847–850.
- Blackburn,E.H. (2001) Switching and signaling at the telomere. *Cell*, **106**, 661–673.
- Blackburn,E.H. and Greider,C.W. (1995) *Telomeres*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Brigati,C., Kurtz,S., Balderes,D., Vidali,G. and Shore,D. (1993) An essential yeast gene encoding a TTAGGG repeat-binding protein. *Mol. Cell Biol.*, **13**, 1306–1314.
- Broccoli,D., Smogorzewska,A., Chong,L. and de Lange,T. (1997) Human telomeres contain two distinct Myb-related proteins, TRF1 and TRF2. *Nat. Genet.*, **17**, 231–235.
- Chikashige,Y. and Hiraoka,Y. (2001) Telomere binding of the Rap1 protein is required for meiosis in fission yeast. *Curr. Biol.*, **11**, 1618–1623.
- Chong,L., van Steensel,B., Broccoli,D., Erdjument-Bromage,H., Hanish,J., Tempst,P. and de Lange,T. (1995) A human telomeric protein. *Science*, **270**, 1663–1667.
- Cooper,J.P., Nimmo,E.R., Allshire,R.C. and Cech,T.R. (1997) Regulation of telomere length and function by a Myb-domain protein in fission yeast. *Nature*, **385**, 744–747.
- Craven,R.J. and Petes,T.D. (1999) Dependence of the regulation of telomere length on the type of subtelomeric repeat in the yeast *Saccharomyces cerevisiae*. *Genetics*, **152**, 1531–1541.
- De Sanctis,V., La Terra,S., Bianchi,A., Shore,D., Burderi,L., Di Mauro,E. and Negri,R. (2002) *In vivo* topography of Rap1p-DNA complex at *Saccharomyces cerevisiae* TEF2 UAS(RPG) during transcriptional regulation. *J. Mol. Biol.*, **318**, 333–349.
- Gilson,E., Laroche,T. and Gasser,S. (1993) Telomeres and the functional architecture of the nucleus. *Trends Cell Biol.*, **3**, 128–134.
- Gottschling,D.E., Aparicio,O.M., Billington,B.L. and Zakian,V.A. (1990) Position effect at *S. cerevisiae* telomeres: reversible repression of Pol II transcription. *Cell*, **63**, 751–762.
- Grossi,S., Bianchi,A., Damay,P. and Shore,D. (2001) Telomere formation by rap1p binding site arrays reveals end-specific length regulation requirements and active telomeric recombination. *Mol. Cell Biol.*, **21**, 8117–8128.
- Henning,K.A., Moskowitz,N., Ashlock,M.A. and Liu,P.P. (1998) Humanizing the yeast telomerase template. *Proc. Natl Acad. Sci. USA*, **95**, 5667–5671.
- Kanoh,J. and Ishikawa,F. (2001) spRap1 and spRif1, recruited to telomeres by Taz1, are essential for telomere function in fission yeast. *Curr. Biol.*, **11**, 1624–1630.
- Karlseeder,J., Smogorzewska,A. and de Lange,T. (2002) Senescence induced by altered telomere state, not telomere loss. *Science*, **295**, 2446–2449.
- Knop,M., Siegers,K., Pereira,G., Zachariae,W., Winsor,B., Nasmyth,K. and Schiebel,E. (1999) Epitope tagging of yeast genes using a PCR-based strategy: more tags and improved practical routines. *Yeast*, **15**, 963–972.
- Koering,C.E., Fourel,G., Binet-Brasselet,E., Laroche,T., Klein,F. and Gilson,E. (2000) Identification of high affinity Tbf1p-binding sites within the budding yeast genome. *Nucleic Acids Res.*, **28**, 2519–2526.
- Krauskopf,A. and Blackburn,E.H. (1996) Control of telomere growth by interaction of RAP1 with the most distal telomeric repeats. *Nature*, **383**, 354–357.
- Krauskopf,A. and Blackburn,E.H. (1998) Rap1 protein regulates telomere turnover in yeast. *Proc. Natl Acad. Sci. USA*, **95**, 12486–12491.
- Kyriou,G., Boakye,K.A. and Lustig,A.J. (1992) C-terminal truncation of RAP1 results in the deregulation of telomere size, stability and function in *Saccharomyces cerevisiae*. *Mol. Cell Biol.*, **12**, 5159–5173.
- Ladenburger,E.M., Keller,C. and Knippers,R. (2002) Identification of a binding region for human origin recognition complex proteins 1 and 2 that coincides with an origin of DNA replication. *Mol. Cell Biol.*, **22**, 1036–1048.
- Li,B., Oestreich,S. and de Lange,T. (2000) Identification of human Rap1: implications for telomere evolution. *Cell*, **101**, 471–483.
- Lin,J.J. and Zakian,V.A. (1996) The *Saccharomyces CDC13* protein is a single-strand TG_{1–3} telomeric DNA-binding protein *in vitro* that affects telomere behavior *in vivo*. *Proc. Natl Acad. Sci. USA*, **93**, 13760–13765.
- Lingner,J., Cooper,J.P. and Cech,T.R. (1995) Telomerase and DNA end replication: no longer a lagging strand problem? *Science*, **269**, 1533–1534.
- Liu,X.S., Brutlag,D.L. and Liu,J.S. (2002) An algorithm for finding protein DNA binding sites with applications to chromatin-immunoprecipitation microarray experiments. *Nat. Biotechnol.*, **20**, 835–839.
- Liu,Z. and Tye,B.K. (1991) A yeast protein that binds to vertebrate telomeres and conserved yeast telomeric junctions. *Genes Dev.*, **5**, 49–59.
- Lustig,A.J. (1992) Hoogsteen G–G base pairing is dispensable for telomere healing in yeast. *Nucleic Acids Res.*, **20**, 3021–3028.
- Marcand,S., Gilson,E. and Shore,D. (1997) A protein-counting mechanism for telomere length regulation in yeast. *Science*, **275**, 986–990.
- Marcand,S., Brevet,V. and Gilson,E. (1999) Progressive *cis*-inhibition of telomerase upon telomere elongation. *EMBO J.*, **18**, 3509–3519.

- McEachern,M.J. and Blackburn,E.H. (1995) Runaway telomere elongation caused by telomerase RNA gene mutations. *Nature*, **376**, 403–409.
- McEachern,M.J., Krauskopf,A. and Blackburn,E.H. (2000) Telomeres and their control. *Annu. Rev. Genet.*, **34**, 331–358.
- Pandita,T.K. (2002) ATM function and telomere stability. *Oncogene*, **21**, 611–618.
- Park,M.J., Jang,Y.K., Choi,E.S., Kim,H.S. and Park,S.D. (2002) Fission yeast Rap1 homolog is a telomere-specific silencing factor and interacts with Taz1p. *Mol. Cells*, **13**, 327–333.
- Pollice,A., Zibella,M.P., Billaud,T., Laroche,T., Pulitzer,J.F. and Gilson,E. (2000) *In vitro* binding of nucleolin to double-stranded telomeric DNA. *Biochem. Biophys. Res. Commun.*, **268**, 909–915.
- Ray,A. and Runge,K.W. (1998) The C terminus of the major yeast telomere binding protein Rap1p enhances telomere formation. *Mol. Cell. Biol.*, **18**, 1284–1295.
- Ray,A. and Runge,K.W. (1999a) Varying the number of telomere-bound proteins does not alter telomere length in tel1Δ cells. *Proc. Natl Acad. Sci. USA*, **96**, 15044–15049.
- Ray,A. and Runge,K.W. (1999b) The yeast telomere length counting machinery is sensitive to sequences at the telomere-nontelomere junction. *Mol. Cell. Biol.*, **19**, 31–45.
- Sandell,L.L. and Zakian,V.A. (1993) Loss of a yeast telomere: arrest, recovery and chromosome loss. *Cell*, **75**, 729–739.
- Schramke,V., Neecke,H., Brevet,V., Corda,Y., Lucchini,G., Longhese,M.P., Gilson,E. and Geli,V. (2001) The set1Δ mutation unveils a novel signaling pathway relayed by the Rad53-dependent hyperphosphorylation of replication protein A that leads to transcriptional activation of repair genes. *Genes Dev.*, **15**, 1845–1858.
- Smogorzewska,A., van Steensel,B., Bianchi,A., Oelmann,S., Schaefer,M.R., Schnapp,G. and de Lange,T. (2000) Control of human telomere length by TRF1 and TRF2. *Mol. Cell. Biol.*, **20**, 1659–1668.
- Strahl-Bolsinger,S., Hecht,A., Luo,K. and Grunstein,M. (1997) SIR2 and SIR4 interactions differ in core and extended telomeric heterochromatin in yeast. *Genes Dev.*, **11**, 83–93.
- Szostak,J.W. and Blackburn,E.H. (1982) Cloning yeast telomeres on linear plasmid vectors. *Cell*, **29**, 245–255.
- Tzfati,Y., Fulton,T.B., Roy,J. and Blackburn,E.H. (2000) Template boundary in a yeast telomerase specified by RNA structure. *Science*, **288**, 863–867.
- van Steensel,B. and de Lange,T. (1997) Control of telomere length by the human telomeric protein TRF1. *Nature*, **385**, 740–743.
- Wach,A., Brachat,A., Pohlmann,R. and Philippsen,P. (1994) New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. *Yeast*, **10**, 1793–1808.
- Wotton,D. and Shore,D. (1997) A novel Rap1p-interacting factor, Rif2p, cooperates with Rif1p to regulate telomere length in *S. cerevisiae*. *Genes Dev.*, **11**, 748–760.
- Zakian,V.A. (1995) Telomeres: beginning to understand the end. *Science*, **270**, 1601–1607.

Received November 18, 2002; revised January 22, 2003;
accepted February 12, 2003