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Rap1 prevents telomere fusions by nonhomologous end joining

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Telomeres protect chromosomes from end-to-end fusions. In yeast *Saccharomyces cerevisiae*, the protein Rap1 directly binds telomeric DNA. Here, we use a new conditional allele of *RAP1* and show that Rap1 loss results in frequent fusions between telomeres. Analysis of the fusion point with restriction enzymes indicates that fusions occur between telomeres of near wild-type length. Telomere fusions are not observed in cells lacking factors required for nonhomologous end joining (NHEJ), including Lig4 (ligase IV), KU and the Mre11 complex. *SAE2* and *TEL1* do not affect the frequency of fusions. Together, these results show that Rap1 is essential to block NHEJ between telomeres. Since the presence of Rap1 at telomeres has been conserved through evolution, the establishment of NHEJ suppression by Rap1 could be universal.

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Introduction

Telomeres are the DNA–protein complexes found at the ends of linear chromosomes. As double-strand ends, telomeres could get involved in DNA double-strand break repair. In most cells, two pathways efficiently repair double-strand breaks: nonhomologous end joining (NHEJ) and homologous recombination. NHEJ is essentially a direct religation between the two ends (Wilson *et al*, 2003). Homologous recombination is a more complex process that uses a template sequence for repair (Symington, 2002). When these pathways act on telomeres, the consequences are utterly different. Homologous recombination events between telomeres can elongate or shorten telomeres but cannot fuse them since telomeric DNA are tandem arrays of short duplex repeats always in the same orientation relative to the chromosome ends. Homologous recombination at telomeres is only partially repressed in normal cells and may play a significant role in telomere length homeostasis, in particular when telomere length has shifted far away from equilibrium (Walmsley *et al*, 1983; Dunn *et al*, 1984; Lundblad and Blackburn, 1993; Li and Lustig, 1996; Le *et al*, 1999; Dunham *et al*, 2000; Teng *et al*, 2000; Cerone *et al*, 2001; Grandin *et al*, 2001; Lustig, 2003; Bailey *et al*, 2004; Londono-Vallejo *et al*, 2004; Tarsounas *et al*, 2004; Teixeira *et al*, 2004; Wang *et al*, 2004). By contrast, a single NHEJ event between two telomeres fuses the involved chromosomes, a gross rearrangement that can initiate a cycle of genomic instability. In normal cells, such event is rare (Ferreira *et al*, 2004). For instance, in wild-type yeast cells, fusions involving a telomere seem to occur in less than 1 every 10^7 cells (DuBois *et al*, 2002; Chan and Blackburn, 2003; Mieczkowski *et al*, 2003). In humans, cytogenetic analyses of normal lymphocytes suggest that chromosome end fusions occur at a frequency lower than 10^{-3} per cell (Prieur *et al*, 1988). Interestingly, end-to-end fusions leading to chain multicentric chromosomes were seen once in lymphocytes from a single patient (Dutrillaux *et al*, 1977). This observation remained exceptional but suggests that dysfunction of NHEJ suppression at telomeres can happen spontaneously in humans.

In fission yeast *Schizosaccharomyces pombe*, the Taz1 protein binds telomeric DNA (Cooper *et al*, 1997). Cells lacking Taz1 accumulate telomere fusions, which required KU and ligase IV, two essential components of the NHEJ machinery (Ferreira and Cooper, 2001, 2004). In mammals, TRF1 and TRF2 are the two telomere-binding proteins orthologous to Taz1 (Chong *et al*, 1995; Bilaud *et al*, 1996, 1997; Broccoli *et al*, 1997). A dominant negative allele of TRF2 displacing the protein from telomeres causes telomere fusions (van Steensel *et al*, 1998). In cells lacking ligase IV, fusions induced by TRF2 loss of function are not observed (Smogorzewska *et al*, 2002). Thus, Taz1 and TRF2 establish NHEJ suppression at telomeres. Taz1 and TRF2 interact at telomeres with a conserved protein, Rap1, which is required for proper telomere length regulation (Li *et al*, 2000; Chikashige and Hiraoka, 2001; Kanoh and Ishikawa, 2001; Li and de Lange, 2003). A role for Rap1 in telomere protection against NHEJ remained to be addressed.

In the budding yeast *Saccharomyces cerevisiae*, there is no ortholog of Taz1/TRF1/TRF2 and Rap1 directly binds the TG_{1-3} telomere sequences (Conrad *et al*, 1990; Konig *et al*, 1996). Rap1 establishes a negative feedback loop on telomere elongation by telomerase (Kyrion *et al*, 1992; Krauskopf and Blackburn, 1996; Marcand *et al*, 1997; Ray and Runge, 1999; Grossi *et al*, 2001; Teixeira *et al*, 2004). For this pathway, Rap1 acts through its carboxy-terminal domain by recruiting two factors, Rif1 and Rif2, whose mode of action is still unknown (Hardy *et al*, 1992; Buck and Shore, 1995; Wotton and Shore, 1997; Levy and Blackburn, 2004; Teixeira *et al*, 2004). Rap1 also establishes transcriptional silencing on the adjacent chromatin by recruiting a different set of factors through the same domain (Kyrion *et al*, 1993; Moretti and Shore, 2001; Luo *et al*, 2002). In addition, Rap1 binds the promoters

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of a large fraction of genes expressed during exponential growth, where it seems to play an essential role in transcriptional activation (Lieb *et al*, 2001). Possibly because of its role in transcription, Rap1 is essential for viability in budding yeast, precluding the use of a simple gene knockout to study its functions (Shore and Nasmyth, 1987).

An indirect approach is to study mutations in the telomerase RNA template that are translated by the recurrent action of telomerase into mutations within the distal repeats of telomeres (Yu *et al*, 1990; Singer and Gottschling, 1994; McEachern and Blackburn, 1995). In budding yeast *Kluyveromyces lactis*, changes in the telomerase RNA template disrupting Rap1 binding site can result in telomere fusions (McEachern *et al*, 2000). This suggests that Rap1 plays a role in telomere protection, although the mutations that abolish Rap1 binding could also impact other pathways. Similar approaches were carried out in *S. cerevisiae* but did not show a requirement for Rap1 in telomere protection against fusions (Prescott and Blackburn, 2000; Alexander and Zakian, 2003; Brevet *et al*, 2003; Lin *et al*, 2004). We addressed this issue differently by looking at the direct consequences of Rap1 protein loss in *S. cerevisiae* using a conditional allele.

Results

Rap1 loss causes telomere fusions

First, we constructed a degron allele of *RAP1*: *rap1*-(Δ). This allele is constructed by the 'double shut off' method and is integrated at the endogenous *RAP1* locus (supplementary data). *rap1*-(Δ) is controlled by the *ANB1* promoter, which is repressible by Rox1. *rap1*-(Δ) encodes a Rap1 protein with an amino-terminal fusion making it a target for Ubr1 and degradation by the N-end rule. Induction of Rox1 and Ubr1 by elevated copper concentration allows the simultaneous transcriptional repression of the gene and proteolysis of the existing molecules. Copper addition to a growing *rap1*-(Δ) cell culture causes Rap1 loss, telomere elongation and cell growth arrest (supplementary data).

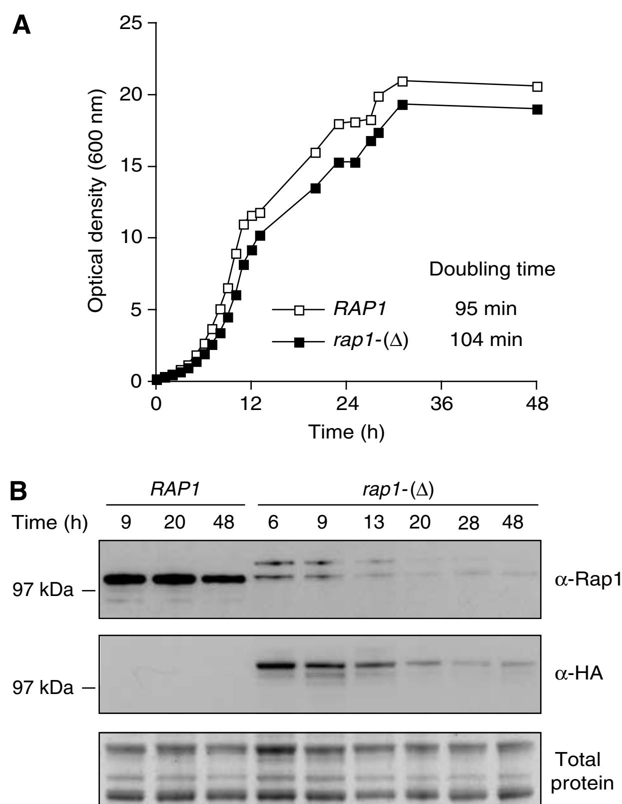

Figure 1 Rap1 loss in *rap1*-(Δ) cells progressing toward stationary phase. (**A**) Growth curve of wild-type and *rap1*-(Δ) cells in rich medium at 30°C. Yeast strains ZMY60 (wild type) and Lev391 (*rap1*-(Δ)) were maintained in exponential phase by successive dilutions in rich medium for 2 days and at time 0 allowed to exhaust the medium. The doubling time is estimated from the initial exponential growth. (**B**) Immunoblot showing Rap1 level in wild-type and *rap1*-(Δ) cells. Rap1 in *rap1*-(Δ) cells is tagged with four HA epitopes. To detect Rap1 and Rap1 tagged with HA epitopes, we used, respectively, a rabbit polyclonal antibody directed against the carboxy-terminal region of Rap1 (α-Rap1) and a monoclonal antibody directed against the HA epitope (α-HA). Loading was controlled by gel staining.

Without copper addition to the medium, *rap1*-(Δ) cells grow exponentially with a slightly higher doubling time and with a lower Rap1 steady-state level than isogenic wild-type cells (Figure 1A and B). This lower amount of Rap1 drops even further when cells exit exponential phase. Telomere length is slightly more heterogeneous in *rap1*-(Δ) cells than in wild type with a mean at 300 and 320 nt, respectively, and remains unchanged during progression toward stationary phase (data not shown). In this study, we used the *rap1*-(Δ) allele in basal conditions (i.e. without copper addition) and took advantage of the protein loss in the mutant to address the contribution of Rap1 to telomere protection.

Fusion events between telomeres were looked at using a PCR strategy similar to the one previously described (Mieczkowski *et al*, 2003). In *S. cerevisiae*, a conserved element, X, is located adjacent to every telomere. In the sequenced yeast genome, 17 out of the 32 chromosome ends display a second element, Y′, inserted between X and the telomere. We chose two primers annealing with X and Y′, respectively, located at about 500 bp from the terminal telomeric repeats (Figure 2A). As shown in Figure 2B, a smearing PCR signal centered at about 1540 bp appears and accumulates in *rap1*-(Δ) cells a few hours after exit from exponential phase, concomitant to Rap1 loss. This signal is not observed in wild-type cells and in *rap1*-(Δ) cells with an integrated copy of the wild-type gene (Figure 2B and C). PCR with only one of the two primers X or Y′ fails to amplify a signal (Figure 2C). This does not rule out fusions between telomeres of the same class (i.e. between two X telomeres and between two Y′ telomeres), which, as palindromes, are likely to resist PCR amplification.

The PCR products were cloned, amplified in *Escherichia coli* and sequenced (Figure 2D). The mean length of the cloned fragments is 1540 ± 120 bp ($n = 83$). All the clones contain an X element end coming from one of 13 different chromosome ends, a Y′ element end on the other side and TG_{1-3} repeats pointing at each other. The quasi-palindromic TG_{1-3} fusions could not be sequenced through the fusion point. To determine the sequence at the junctions, we looked for restriction sites that would be formed by joining inverted TG_{1-3} repeats. Tested sites were found with occurrences not statistically different from those expected from a random fusion between two telomeres (Table I). Among the clones with a restriction site at the junction, the mean length of the telomeres trapped in the fusion is 240 ± 80 bp ($n = 58$).

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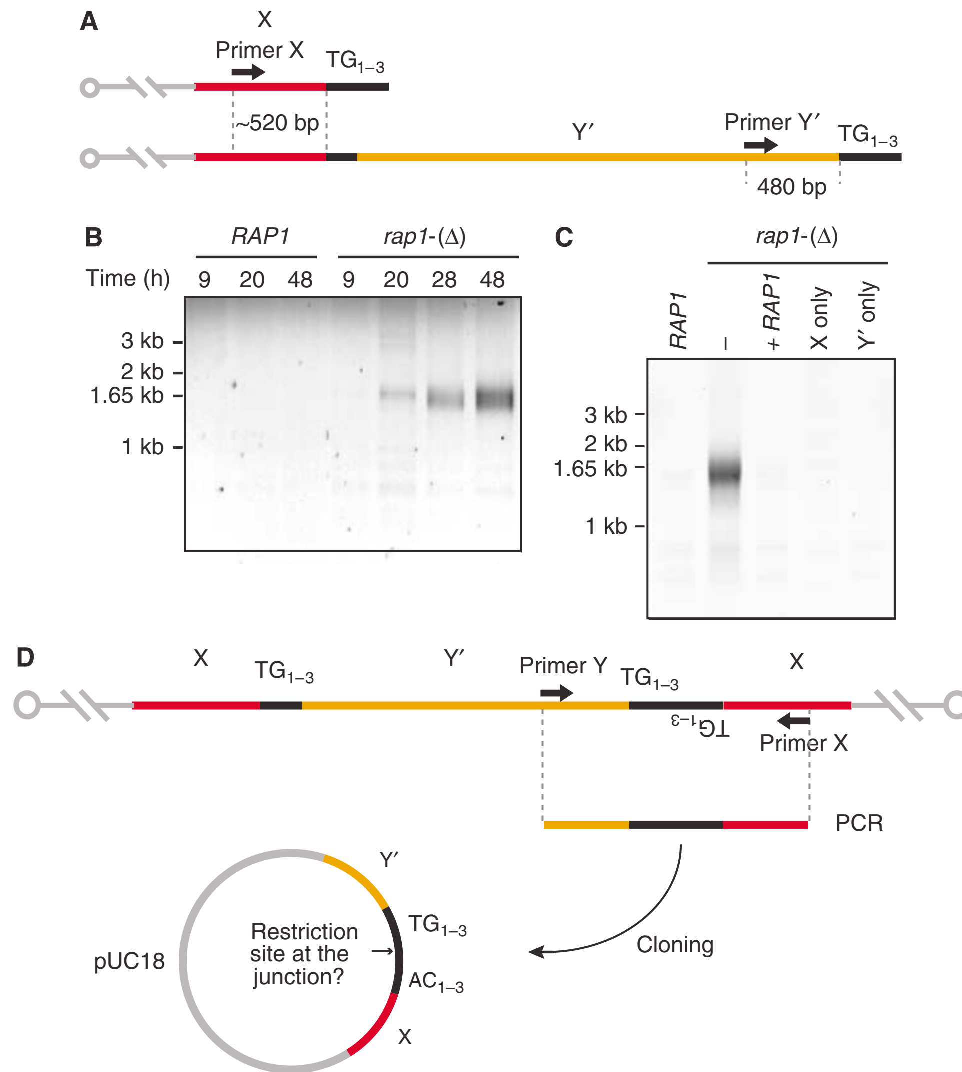

Figure 2 Telomere fusions in *rap1*-(Δ) cells progressing toward stationary phase. (**A**) Schematic representation of X and Y′ telomeres in *S. cerevisiae* and relative positions of the primers used for PCR amplification. In the sequenced yeast genome, there are 15 X telomeres and 17 Y′ telomeres. The fusion of two telomeres should give a PCR product of about 1000 bp plus the TG_{1-3} telomeric repeats at the junction. Fusions between two X or two Y′ telomeres form quasiperfect palindromes and are unlikely to be amplified. (**B**) Detection by PCR of telomere fusions in *rap1*-(Δ) cells reaching stationary phase. Time points are the same as in Figure 1. (**C**) Complementation of *rap1*-(Δ) in Lev391 with a wild-type copy of *RAP1* integrated at *URA3*. Cells were grown to saturation in rich medium for 5 days. Telomere fusions are not amplified with only one of the two primers X or Y′. (**D**) Schematic representation of PCR amplification, cloning and restriction analysis of telomere fusions.

Table I Restriction site occurrences at the junction among the cloned fusions

Enzyme name	Enzyme site	Occurrence	Expected[a]
*Hpy*8I	-GTNNAC-	28	27.9
*Rsa*I	-GTAC-	6	11.6
*Apa*LI	-GTGCAC-	10	11.6
*Kpn*I	-GGTACC-	1	1.8
Total number of clones		83	

[a]The expected occurrences are calculated with a native 350 bp telomeric sequence displaying a GC content of 62%.

Together, these results show that telomeres of near wild-type length fuse with each other when Rap1 disappears in *rap1*-(Δ) cells.

The *rap1*-(Δ) allele expresses a protein with an amino-terminal tag. Telomere fusions observed in *rap1*-(Δ) cells could be a consequence of Rap1 loss, Rap1 tagging or both. To address this, we used a strain in which *RAP1* is simply deleted and viability rescued by an ectopic copy of the wild-type gene. The *RAP1* sequence is either on a plasmid or integrated in a chromosome (Figure 3A). Among these cells, telomere fusions occur when *RAP1* is on a plasmid but not when *RAP1* is integrated (Figure 3B). We propose that the fusions are caused by continuous loss of the plasmid among the growing cell population, producing doomed cells that lose wild-type Rap1, indicating that Rap1 loss is sufficient to cause telomere fusions. The PCR used to detect telomere fusions is semiquantitative. We estimated that fusions are about 80 times more frequent in *rap1*-(Δ) cells than in *rap1*-Δ cells with *RAP1* on a plasmid (Figure 3C). In the latter situation, *RAP1* is lost only in a small fraction of cells and the wild-type protein is itself more stable, probably explaining why the fusions are less frequent.

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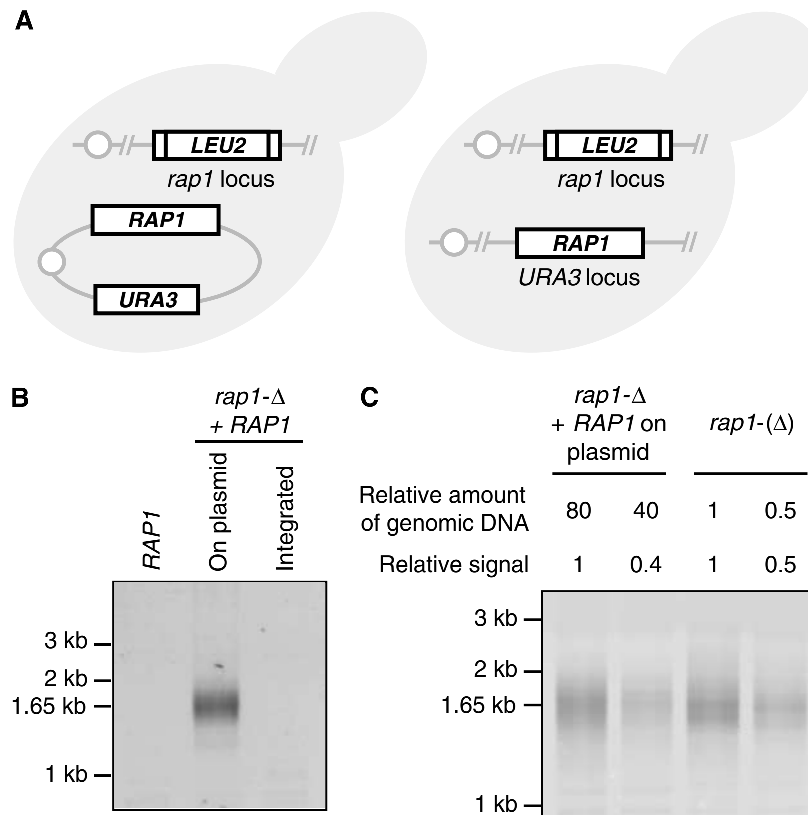

Figure 3 Telomere fusions among cells with *RAP1* on a plasmid. (**A**) Schematic representation of the yeast strains deleted for *RAP1* and carrying a wild-type copy of *RAP1* either on a plasmid or integrated in a chromosome. (**B**) Continuous loss of wild-type *RAP1* gene on a plasmid causes telomere fusions. In the yeast strain Lev9, plasmid pCEN-Sup4-RAP1 was replaced by centromeric plasmid pRS316-RAP1 or by pRS306-RAP1 integrated at *URA3*. The control *RAP1* strain is W303-1a. Cells were grown to saturation in rich medium for 5 days. Telomere fusions were amplified by PCR. (**C**) Relative quantification of telomere fusions. Strains Lev9 shuffled with pRS316-RAP1 and Lev391 (*rap1*-(Δ)) were grown to saturation in rich medium for 5 days. Fusions were amplified by PCR and quantified as described in Materials and methods.

Formally, Rap1 could establish two different types of mechanisms: it could prevent the fusions from occurring and it could revert telomere fusions once they have occurred. We looked at the outcome of a telomere fusion once it is formed. A fusion was cloned from *rap1*-(Δ) cells into a centromeric yeast plasmid and reintroduced into wild-type cells. Southern analysis shows that this fusion on a circular plasmid can be maintained for many generations prior to rearrangements within the fusion or linearization of the plasmid (data not shown). This relative stability suggests that Rap1 must act before the fusion to prevent it.

Telomere fusions are produced by NHEJ

NHEJ requires the DNA end-binding proteins Yku70 and Yku80 that form the KU heterodimer, as well as the ligase Lig4 and its associated factor Lif1 (ligase IV and XRCC4 respectively in mammals) (Milne *et al*, 1996; Schar *et al*, 1997; Teo and Jackson, 1997; Wilson *et al*, 1997; Herrmann *et al*, 1998; Lee *et al*, 1998; Teo and Jackson, 2000; Chen *et al*, 2001; Walker *et al*, 2001). In addition, at least in *S. cerevisiae*, NHEJ required the Lif1-interacting factor Lif2 (also called Nej1) and the Mre11 complex made of Mre11, Rad50 and Xrs2 (Moore and Haber, 1996; Boulton and Jackson, 1998; Frank-Vaillant and Marcand, 2001; Kegel *et al*, 2001; Ooi *et al*, 2001; Valencia *et al*, 2001). To address the pathway responsible for fusing Rap1-defective telomeres, we tested the deletion of genes required for NHEJ. As shown in Figure 4, in *rap1*-(Δ) cells lacking Yku70, Yku80, Lig4, Lif1, Lif2, Mre11, Rad50 and Xrs2, telomere fusions could not be detected by PCR. Complementation of the *lif2* disruption by a centromeric plasmid encoding Lif2 restores the appearance of telomere fusions in *rap1*-(Δ) cells (Figure 4A). Thus, telomere fusions caused by Rap1 loss are produced by NHEJ.

We examined the involvement in telomere fusion of two known regulators of the Mre11 complex that could affect the processing of telomere ends, thereby influencing fusions by NHEJ. Sae2 is an activator of the Mre11 nuclease activity (Rattray *et al*, 2001; Lobachev *et al*, 2002). Tel1 is the yeast ortholog of the human DNA damage checkpoint kinase ATM and is required for the function of the Mre11 complex in telomere elongation by telomerase (Greenwell *et al*, 1995; Ritchie and Petes, 2000; Tsukamoto *et al*, 2001). *tel1*-Δ cells display short telomeres (Greenwell *et al*, 1995). As shown in Figure 4B, Sae2 and Tel1 are not required for fusion in *rap1*-(Δ) cells. As expected for fusions between shorter telomeres, *rap1*-(Δ) *tel1*-Δ double mutant cells display shorter fusion products.

In mammals, the ERCC1-XPF nuclease is required for the telomere fusions induced by TRF2 inhibition (Zhu *et al*, 2003). The yeast homolog of ERCC1-XPF is Rad1-Rad10. In a *rap1*-(Δ) *rad1*-Δ double mutant, telomere fusions are still observed but the double mutant displays a significant growth defect compared to *rap1*-(Δ) and *rad1*-Δ single mutants (data not shown). This negative genetic interaction is surprising and will require further investigation to be explained.

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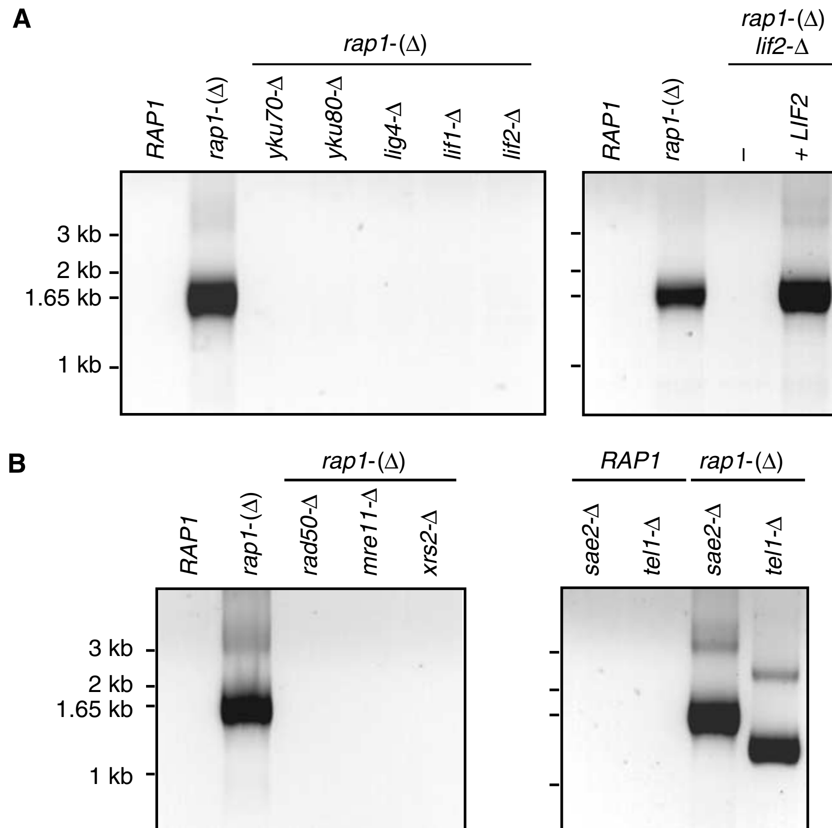

Figure 4 NHEJ factors are required for telomere fusions. (**A**) Yeast strains ZMY60 (wild type), Lev391 (*rap1*-(Δ)), Lev397 (*rap1*-(Δ) *yku70*-Δ), Ybp43 (*rap1*-(Δ) *yku80*-Δ), Ybp11 (*rap1*-(Δ) *lig4*-Δ), Lev396 (*rap1*-(Δ) *lif1*-Δ) and Ybp14 (*rap1*-(Δ) *lif2*-Δ) were grown to saturation in rich medium for 7 days. Strains ZMY60, Lev391 and Ybp14 transformed with plasmid pRS314 and strain Ybp14 transformed with plasmid pRS314-LIF2 were grown to saturation in synthetic medium lacking tryptophan for 5 days. Telomere fusions were amplified by PCR. (**B**) Yeast strains ZMY60, Lev391, Ybp29 (*rap1*-(Δ) *mre11*-Δ), Ybp27 (*rap1*-(Δ) *rad50*-Δ), Ybp9 (*rap1*-(Δ) *xrs2*-Δ), Ybp40 (*sae2*-Δ), Ybp41 (*tel1*-Δ), Ybp31 (*rap1*-(Δ) *sae2*-Δ) and Ybp23 (*rap1*-(Δ) *tel1*-Δ) were grown to saturation in rich medium for 7 days. Telomere fusions were amplified by PCR.

To estimate the absolute frequencies of fusions, a centromeric plasmid with a fusion cloned from *rap1*-(Δ) cells was reintroduced into wild-type cells (Figure 5A). Its average copy number was estimated by Southern analysis at about 1.9 copies per genome (data not shown). The increase in copy number is probably a consequence of the antagonism between the telomere sequences and the centromere (Enomoto *et al*, 1994). The signal from this plasmid provides an external control for quantification by PCR of the fusions in *rap1*-(Δ) and *rap1*-(Δ) *sae2*-Δ cells. To quantify fusions in *rap1*-(Δ) *tel1*-Δ cells, a second plasmid with a short fusion cloned from *rap1*-(Δ) *tel1*-Δ cells was used. This plasmid is maintained at about 1.3 copies per genome (data not shown). As shown in Figure 5, in cells in stationary phase, we observe about one fusion between an X telomere and a Y′ telomere every two genomes. The number of fusions is not significantly different in the absence of Sae2 or Tel1 (Figure 4B and C). Since fusions can presumably also occur between telomeres of the same class, we probably underestimate the actual frequencies of fusions by about two-fold.

Telomere fusions reduce cell viability

These relatively high frequencies of fusions per cell prompted us to look for an impact on cell viability. Cells were grown to saturation in liquid media for 3–4 weeks and individually placed on plates. When each cell resumed growth, the two products of its first division were separated and one was placed at an adjacent position (Figure 6A). Then, each cell was allowed to form a colony. Four different outcomes are observed and represented in Figure 6B: (a) the products of the first division give rise to two viable colonies, (b) the initial cell fails to re-enter the cell cycle or to finish the first division, (c) only one of the two products of the first division gives rise to a viable colony and, finally, (d) the two products of the first division fail to produce a viable colony. In this one-generation pedigree, we observed that, in a wild-type strain, a majority of cells form two viable colonies, about a third failed to resume growth and only a small fraction produced unviable microcolonies (Figure 6D). Compared to the wild-type strain, the *rap1*-(Δ) strain displays a higher frequency of cells that re-entered the cell cycle and failed to form viable colonies and increased asymmetric lethality, when only one of the two products of the first division gives rise to a viable colony. The *rap1*-(Δ) *lif1*-Δ double mutant defective for NHEJ behaves as the wild-type strain (Figure 6D), suggesting that increased failure to form a viable colony in *rap1*-(Δ) cells is a consequence of telomere fusions.

To explain asymmetric lethality, we propose the following scenario. A dicentric chromosome, formed by a telomere fusion in stationary phase and duplicated in S phase, will break during anaphase if the centromeres present on the same chromatid are pulled apart to opposite poles. Two breaks, one on each sister chromatid, will occur. Irrespective of the break positions, one cell will have lost at

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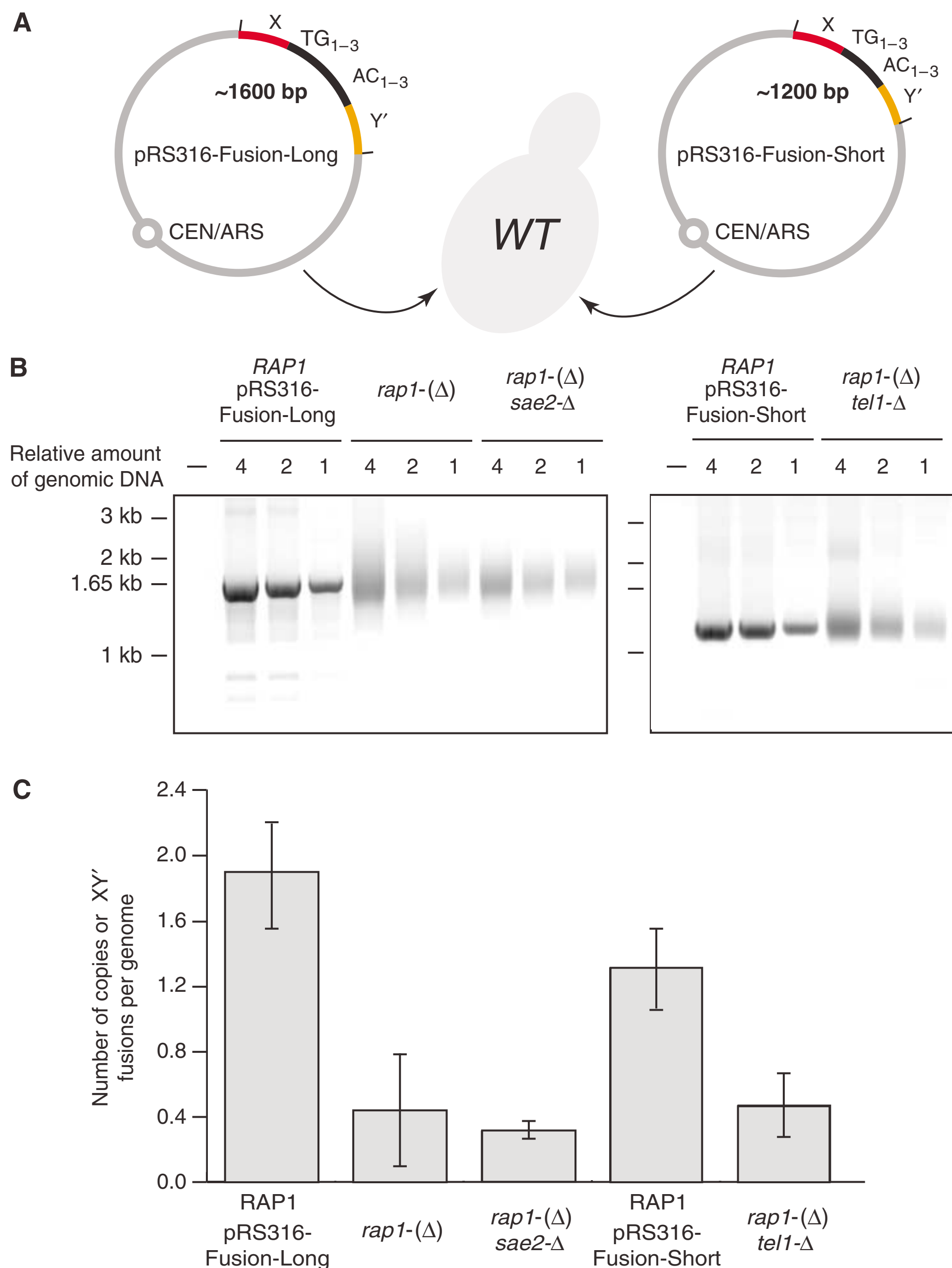

Figure 5 Quantification of telomere fusions. (**A**) Schematic map of the pRS316-Fusion-Long and pRS316-Fusion-Short plasmids. In pRS316-Fusion-Long, the X element comes from the right end of chromosome II. An *Apa*LI site is present at the junction and the two TG_{1-3} repeats pointing at each other are about 350 and 270 bp. In pRS316-Fusion-Short, the X element comes from the right end of chromosome IX. The sum of the TG_{1-3} inverted repeats is about 200 bp. pRS316-Fusion-Long and pRS316-Fusion-Short were separately reintroduced into wild-type cells. (**B**) Quantification of fusions between X and Y′ telomeres. Yeast strains ZMY60 (wild type) transformed with pRS316-Fusion-Long and pRS316-Fusion-Short were grown to saturation for 5 days in medium lacking uracil. Lev391 (*rap1*-(Δ)), Ybp31 (*rap1*-(Δ) *sae2*-Δ) and Ybp23 (*rap1*-(Δ) *tel1*-Δ) were grown to saturation for 5 days in rich medium. Fusions were amplified by PCR and quantified as described in Materials and methods. (**C**) Number of fusions per genome. Plasmid copy number was estimated by Southern analysis (data not shown). The signals from strains Lev391 (*rap1*-(Δ)) and Ybp31 (*rap1*-(Δ) *sae2*-Δ) were normalized with the signals from strain ZMY60 (wild type) transformed with pRS316-Fusion-Long. The signals from strain Ybp23 (*rap1*-(Δ) *tel1*-Δ) were normalized with the signals from strain ZMY60 (wild type) transformed with pRS316-Fusion-Short. Mean and standard deviation were calculated by averaging 12 amplifications (three dilutions of four independent samples).

least one chromosome fragment and will be unlikely to form a viable colony. The other cell will receive a complete set of genes and maintain the possibility of forming a viable colony. This could occur through repair by break-induced replication (BIR) until the fusion is resolved, for instance through degradation from a break up to the fused telomeres. If during the first division the two centromeres present on the same chromatid go toward the same pole, the two cells will inherit the dicentric chromosome. Breakage will be postponed to later divisions, increasing the probability of giving rise to viable colonies. If more than one fusion is formed in stationary phase, the possibility of a viable outcome will be significantly reduced, explaining that the two products of the first division can fail to form viable colonies. In conclusion,

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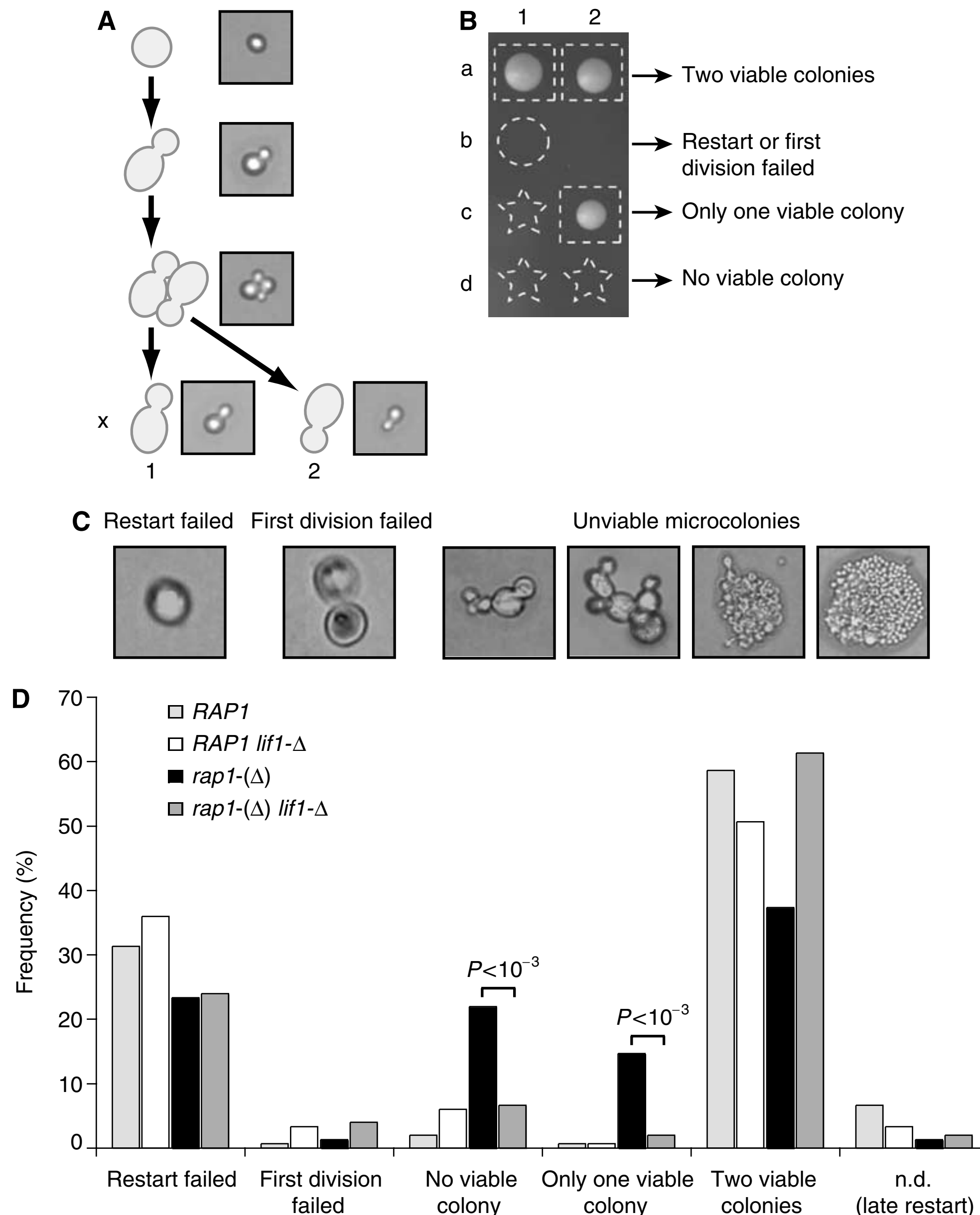

Figure 6 Telomere fusions reduce cell viability. (**A**) Schematic representation of single cell micromanipulation during its first division. Yeast strains were grown to saturation in rich medium for 3–4 weeks. Cells were spread onto plates, micromanipulated onto a grid and left at 30°C. The two products of the first division were separated as budded cells and placed adjacent to each other on the same line. An example has been recorded at each step and is shown. (**B**) After micromanipulation, cells were grown for 4 days at 30°C. Possible outcomes are shown. Dashed rectangles denote the cell that gave rise to a viable colony, the dashed circle denotes the initial cell that failed to restart growth or achieve its first division and dashed stars denote the cell that failed to achieve its first division or formed an unviable microcolony of two to a few hundred cells. (**C**) Morphology of cells that failed to form a viable colony. (**D**) Cell outcomes after stationary phase. Yeast strains ZMY60 (wild type), Lev391 (*rap1*-(Δ)), Lev396 (*rap1*-(Δ) *lif1*-Δ) and Lev398 (*lif1*-Δ) were grown and micromanipulated as described in panel A. The data come from two independent experiments ($n_{\mathrm{total}} = 150$ cells). *P*-values were calculated using a Student's test.

telomere fusions occurring in stationary phase reduce cell viability when cells resume cell division, but a single telomere fusion in a haploid cell does not seem to be necessarily a lethal event.

Discussion

We describe here that Rap1 loss in cells exiting the cell cycle results in telomere fusions by NHEJ, indicating that Rap1 prevents this pathway at telomeres. The timing of Rap1 loss in *rap1*-(Δ) mutant cells separates the functions of Rap1. Indeed, another function of Rap1 at telomeres is to negatively regulate telomere elongation by telomerase (Kyrion *et al*, 1992; Marcand *et al*, 1997). Telomere elongation is restricted to late S phase when telomeres are replicated (Marcand *et al*, 2000; Taggart *et al*, 2002). By causing Rap1 loss in cells that do not progress through S phase, the *rap1*-(Δ) mutant allows the progressive accumulation of fusions between telomeres that remain at normal lengths. In addition, Rap1 involvement in transcriptional activation during exponential growth is probably relieved when cells are out of the cell cycle as suggested by the ability of *rap1*-(Δ) cells to normally enter

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and exit stationary phase. Thus, Rap1 role on NHEJ suppression can be studied in cells that are not grossly challenged in housekeeping genes expression.

In yeast, in the absence of telomerase, telomeres get progressively very short and fuse by NHEJ at increasing frequencies (Chan and Blackburn, 2003; Mieczkowski *et al*, 2003). We propose that in this situation fusions occur when the remaining Rap1 binding sites become insufficient to insure full NHEJ inhibition. Interestingly, Tel1 loss in cells lacking telomerase increases the frequency of fusions (Chan and Blackburn, 2003). Here we show that a *tel1*-Δ mutant does not affect fusions caused by Rap1 loss (Figure 5). It suggests that, among the mechanisms established by Rap1 to suppress NHEJ, one involves Tel1. Another explanation would be that Tel1 loss alleviates a checkpoint and in the absence of telomerase increases the frequency of critically short telomeres that can be subjected to NHEJ, a situation that would not happen when telomerase is present as in *rap1*-(Δ) cells.

It would be interesting to know what is the minimum number of Rap1 molecules sufficient to establish NHEJ suppression at telomeres. Rap1 binds telomeric DNA at a density of 1 per 18 bp (Gilson *et al*, 1993). In *tel1*-Δ cells, telomere length is between 100 and 150 bp, which corresponds to five to nine Rap1 binding sites, and telomeres do not seem to fuse (DuBois *et al*, 2002; Mieczkowski *et al*, 2003). A study by Chan and Blackburn (2003) shows that, in telomerase-defective cells with a mean telomere length of 230 bp, fusions involve very short telomeres of about 33 bp, that is two Rap1 binding sites. This strong bias suggests that three Rap1 molecules are enough to repress NHEJ significantly.

Our results do not directly address the mechanism(s) by which Rap1 establishes NHEJ suppression at telomeres. KU is constitutively bound to telomeres (Gravel *et al*, 1998), suggesting that Rap1 or factors recruited by Rap1 act downstream of KU binding to inhibit the synapsis, processing and ligation steps of NHEJ. This could be mediated through the formation of a DNA secondary structure that escapes NHEJ, as previously suggested in higher eukaryotes (Griffith *et al*, 1999; Zhu *et al*, 2003), and through protein–protein interactions. The experimental approach presented here should allow us to address these models in budding yeast.

In fission yeast *S. pombe* and in mammals, Rap1 is present at telomeres through interactions with the telomeric DNA binding factors Taz1 and TRF2, respectively (Li *et al*, 2000; Chikashige and Hiraoka, 2001; Kanoh and Ishikawa, 2001; Li and de Lange, 2003). In *S. pombe*, the absence of Rap1 results in telomere fusions by NHEJ (Miller *et al*, 2005). Thus, despite differences in the assembly of telomeric protein–DNA complexes between budding yeasts and other eukaryotes, Rap1 role in establishing NHEJ inhibition at telomeres appears to be conserved. Understanding how Rap1 acts will be relevant to the biology of human telomeres.

Materials and methods

Amplification and analysis of telomere–telomere fusions

Telomere fusions were amplified by PCR. Genomic DNA was prepared by phenol–chloroform extraction and resuspended in TE pH 8.0 buffer. One primer (X; AGGGTATAGACCGCTGAGGCAAGTG) had a sequence from X elements (e.g. coordinates 649–673 of chromosome IV; Stanford Genome Database) and the second (Y′; AGCGATGATGTTCCAGACGGTAGAT) had a sequence from Y′ elements (e.g. coordinates 314–338 of chromosome V). PCR reactions (30 μl) contained genomic DNA ~10 ng, ProofStart buffer 1× supplemented with $MgSO_4$ 0.83 mM, dNTP 0.3 mM each, primers 1 μM each, HotStarTag 1.2 U (Qiagen™) and ProofStart 0.12 U (Qiagen™). The conditions were as follows: 95°C 15 min; then 25–30 cycles of 94°C 30 s, 68°C 30 s, 72°C 1 min 30 s; followed by 72°C 3 min. The products were run through a 1% agarose gel and visualized by ethidium bromide staining. Amplified fusions were cloned by *Hin*dIII–*Eco*RI digestion into pUC18 using primers with added restriction sites. The clones were amplified in XL1-blue cells grown at 25°C, analyzed by restriction and sequenced. The restriction analysis for *Hpy*8I, *Rsa*I, *Apa*LI and *Kpn*I was facilitated by the X and Y′ sequence alignments provided by Dr E Louis at http://www.le.ac.uk/genetics/ejl12/index.html.

Semiquantitative PCR

Quantifications were carried out with the same PCR conditions. The relevant genomic DNA was diluted with genomic DNA from a wild-type strain to maintain the total DNA concentration constant at ~10 ng. The products were run through a 1% agarose gel containing 0.1 μg/ml ethidium bromide and fluorescence was quantified using a Typhoon™ imager and ImageQuant™ 5.0 software (Amersham Biosciences™). The PCR used to detect fusions remains sensitive at at least three orders of magnitude below the level of fusions observed in *rap1*-(Δ) cells (data not shown).

Strains and plasmids

The strains used in this study are listed in Table II. The *rap1*-(Δ) allele was generated in strain ZMY60 by PCR-mediated transformation using the method described by Dubacq *et al* (2002). Gene deletions were made by PCR-mediated transformation (Longtine *et al*, 1998).

The *RAP1* gene (a *Sac*I–*Xba*I genomic fragment) was inserted into pRS306 (integrative, *URA3*) and pRS316 (*CEN, URA3*) creating plasmids pRS306-RAP1 and pRS316-RAP1, respectively. pRS306-RAP1 was digested by *Nde*I prior to transformation into yeast. A telomere fusion that was cloned into pUC18 and analyzed was subcloned using *Eco*RI and *Hin*dIII into pRS316 to create plasmid pRS316-Fusion. The *LIF2* gene (including 360 bp upstream of the start codon and 330 bp downstream of the stop codon) was inserted into pRS314 (*CEN, TRP1*) creating plasmid pRS314-LIF2.

Telomere length

Telomere length in wild-type and *rap1*-(Δ) cells was measured by PCR after end labeling with terminal transferase as described (Teixeira *et al*, 2004). The mean length of the X telomeres was 320

Table II Yeast strains used in this study

Strain	Genotype
ZMY60 (Moqtaderi *et al*, 1996)	*MAT**a** ura3-52 trp1-Δ1 ade2-101 pACE1-UBR1 pACE1-ROX1*
Lev391	ZMY60 *rap1*-(Δ)::*KAN*^{r} (*KAN*^{r}*-ANB-UB-R-lacI-4HA-RAP1)*
Lev397	Lev391 *yku70*-Δ::*klURA3*
Ybp43	Lev391 *yku80*-Δ::*klURA3*
Ybp11	Lev391 *lig4*-Δ::*klURA3*
Lev396	Lev391 *lif1*-Δ::*klURA3*
Ybp14	Lev391 *lif2*-Δ::*klURA3*
Ybp29	Lev391 *mre11*-Δ::*klURA3*
Ybp27	Lev391 *rad50*-Δ::*klURA3*
Ybp9	Lev391 *xrs2*-Δ::*klURA3*
Ybp40	ZMY60 *sae2*-Δ::*klURA3*
Ybp41	ZMY60 *tel1*-Δ::*klURA3*
Ybp31	Lev391 *sae2*-Δ::*klURA3*
Ybp23	Lev391 *tel1*-Δ::*klURA3*
Lev398	ZMY60 *lif1*-Δ::*klURA3*
W303-1a	*MAT**a** ade2-1 trp1-1 ura3-1 leu2-3,112 his3-11,15 can1-100 rad5-535*
YLS85 (Sussel and Shore, 1991)	W303-1a *rap1*::*LEU2 pCEN-Sup4-RAP1*

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and 330 nt in wild-type and *rap1*-(Δ) cells, respectively. The mean length of the Y′ telomeres was 320 and 270 nt in wild-type and *rap1*-(Δ) cells, respectively (data not shown). The mean length of the telomeres in the cloned fusions with a restriction site at the junction was 270±80 bp ($n = 29$) on the X side and 220±70 bp on the Y′ side ($n = 29$).

Western blot

Proteins were extracted at 95°C in urea 8 M, SDS 2% and Tris–HCl pH 7.5 100 mM followed by vortexing at 2500 r.p.m. with glass beads. A 10 μg portion of proteins was run through a 4–12% NuPage gel (Invitrogen™) and blotted onto a nitrocellulose membrane. To detect Rap1 and Rap1 tagged with HA epitopes, we used, respectively, a rabbit polyclonal antibody directed against the carboxy-terminal region of Rap1 (sc-20167; SantaCruz™; 1:500) and a monoclonal antibody directed against the HA epitope (12CA5; 1:10 000). Loading was controlled by gel staining with *Simple Blue*™ (Invitrogen™).

Supplementary data

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

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