A novel Rap1p-interacting factor, Rif2p, cooperates with Rif1p to regulate telomere length in *Saccharomyces cerevisiae*

David Wotton¹ and David Shore^{2,3}

Department of Microbiology, Columbia University College of Physicians and Surgeons, New York, New York 10032 USA;

²Department of Molecular Biology, University of Geneva, CH-1211 Geneva 4, Switzerland

The *Saccharomyces cerevisiae* Rap1 protein binds with high affinity to sites within the poly(C₁₋₃A) tracts at telomeres, where it plays a role in both telomere length regulation and the initiation of telomeric silencing. Rap1p initiates silencing at telomeres by interacting through its carboxy-terminal domain with Sir3p and Sir4p, both of which are required for repression. This same domain of Rap1p also negatively regulates telomere elongation, through an unknown mechanism. We have identified a new Rap1-interacting factor (Rif2p) that plays a role in telomere length regulation. Rif2p has considerable functional similarities with a Rap1-interacting factor (Rif1p) identified previously. Mutations in *RIF1* or *RIF2* (unlike mutations in the silencing genes *SIR3* and *SIR4*) result in moderate telomere elongation and improved telomeric silencing. However, deletion of both *RIF1* and *RIF2* in the same cell results in a dramatic increase in telomere length, similar to that seen with a carboxy-terminal truncation of Rap1p. In addition, overexpression of either *RIF1* or *RIF2* decreases telomere length, and co-overexpression of these proteins can reverse the telomere elongation effect of overexpression of the Rap1p carboxyl terminus. Finally, we show that Rif1p and Rif2p can interact with each other in vivo. These results suggest that telomere length regulation is mediated by a protein complex consisting of Rif1p and Rif2p, each of which has distinct regulatory functions. One role of Rap1p in telomere length regulation is to recruit these proteins to the telomeres.

[Key Words: Rap1p; Rif1p; Rif2p; telomere length; transcriptional silencing; *Saccharomyces cerevisiae*]

Received November 4, 1996; revised version accepted January 31, 1997.

Telomeres, the ends of eukaryotic chromosomes, are specialized protein-DNA complexes typically based upon a simple DNA-repeat structure (TTAGGG in many multicellular organisms). Telomeres in the yeast *Saccharomyces cerevisiae*, which have an irregular repeat structure commonly abbreviated as C₁₋₃A, are essential for chromosome stability (Sandell and Zakian 1993). The maintenance of telomeres in yeast (Singer and Gottschling 1994), and probably most other organisms, requires a unique reverse transcriptase, called telomerase, that can add telomeric repeats onto the chromosome ends (for review, see Blackburn 1994; Zakian 1995b). The length of telomeric repeats is variable between strains and individual clones of a given strain and can spontaneously increase or decrease (Shampay and Blackburn 1988), suggesting a balance between telomerase addition and degradation that is under complex genetic control

(Lustig and Petes 1986; for review, see Greider 1996; Zakian 1995a).

The telomeric repeats in *S. cerevisiae* form high-affinity binding sites for the essential repressor/activator protein 1 (Rap1p) (Shore and Nasmyth 1987; Buchman et al. 1988; Longtine et al. 1989) as often as once per 18 bp (Gilson et al. 1993). Rap1p plays two roles at telomeres, both of which are mediated by its carboxy-terminal domain (for review, see Shore 1995; Zakian 1995a): It is involved in transcriptional silencing, or telomere position effect (TPE) (Kyrion et al. 1993; Moretti et al. 1994), and it controls telomere length (Conrad et al. 1990; Lustig et al. 1990; Sussel and Shore 1991; Kyrion et al. 1992; Liu et al. 1994). Rap1p is also involved in silencing at the *HM* mating-type loci (Kurtz and Shore 1991; Sussel and Shore 1991; Kyrion et al. 1993). The role of Rap1p in transcriptional silencing appears to be the recruitment of specific repressor proteins (Sir3p and Sir4p) to the telomeres and *HM* silencers via direct protein-protein interactions (Moretti et al. 1994; Cockell et al. 1995; Hecht et al. 1996). In addition to its specialized roles at telomeres and *HM* silent mating-type loci, Rap1p also binds to the

¹Present address: Memorial-Sloan Kettering Cancer Center, Cell Biology and Genetics, New York, New York 10021 USA.

³Corresponding author.

E-MAIL: David.Shore@molbio.unige.ch; FAX 41 22 702 68 68.

upstream regions of many genes, including a large number of ribosomal protein and glycolytic enzyme genes, where the protein appears to function as a transcriptional activator (for review, see Shore 1994). Activation of transcription by Rap1p may be mediated by a region immediately amino-terminal to the silencing and telomere domain (Hardy et al. 1992a) and involves, at least in some cases, specific interactions with other DNA-binding activator proteins (Tornow et al. 1993; Drazinic et al. 1996).

Point mutations (e.g., *rap1^s*) and frameshift mutations (e.g., *rap1^t*) within the Rap1p carboxyl terminus result in varying degrees of telomere tract elongation, suggesting that this domain regulates telomere elongation (Sussel and Shore 1991; Kyriou et al. 1992; Liu et al. 1994). However, this function of Rap1p does not appear to be mediated directly by the Sir proteins. In fact, mutation of *SIR3* or *SIR4* actually results in slight telomere shortening (Palladino et al. 1993), suggesting that the normal function of these proteins in some way supports telomere elongation. Instead, a number of studies indicate that other proteins interact with the Rap1p carboxyl terminus to negatively regulate telomere growth. For example, overexpression of the Rap1p carboxyl terminus in the absence of its centrally located DNA-binding domain (Conrad et al. 1990; Hardy 1991) or introduction of extra telomere repeats into cells (Runge and Zakian 1989) results in telomere elongation, consistent with the idea that titratable Rap1-interacting factors control telomere length. One candidate for such a factor is Rif1p, which was identified in a two-hybrid screen as a protein that interacts with the Rap1p carboxyl terminus (Hardy et al. 1992b). Interestingly, the telomere elongation defect of *rap1^s* mutants may result from an inability to bind Rif1p: Mutant *rap1^s* proteins interact weakly or not at all with Rif1p in two-hybrid assays, and disruption of *RIF1* results in telomere elongation similar to that observed in severe *rap1^s* mutants (Sussel and Shore 1991; Hardy et al. 1992b). However, several lines of evidence point to the existence of other Rap1p-interacting factors involved in telomere length regulation. First, *rap1^t* mutants (which completely lack the carboxyl terminus) have a much more severe telomere elongation phenotype than either *rap1^s* or *rif1* mutants (Kyriou et al. 1992). Second, point mutations in the Rap1p carboxyl terminus with weak telomere elongation phenotypes display additive effects when present together or in combination with a *RIF1* mutation (Liu et al. 1994; L. Sussel, S. Buck, and D. Shore, unpubl.). Finally, overexpression of the Rap1 carboxyl terminus causes further telomere elongation in *RIF1* mutant cells, suggesting the existence of other Rap1p-interacting factors that regulate telomere length (Wiley and Zakian 1995).

Here we present the identification and characterization of a novel protein, which we call Rif2p (Rap1-interacting factor 2p). This protein interacts with the carboxyl terminus of Rap1p in the two-hybrid system, and is also able to interact with Rif1p. Rif2p has striking functional similarities to Rif1p, despite a lack of sequence similarity. However, deletion of both *RIF* genes has a strong

synergistic effect on telomere length, resulting in telomere elongation similar to that seen in *rap1^t* cells. We argue from these and other data that Rif1p and Rif2p form a functional complex capable of regulating telomere length when recruited to telomeres by Rap1p.

Results

Isolation of RIF2

To identify proteins that interact with Rap1p and play a role in mediating Rap1p-dependent functions, we have used the two-hybrid system to screen yeast genomic DNA libraries with a LexA/Rap1 fusion encoding amino acids 635–827 of Rap1p. Previously we reported the isolation of clones containing *SIR3* and *SIR4*, two genes directly involved in transcriptional silencing (Moretti et al. 1994). Here we describe the isolation and characterization of a new gene from this two-hybrid screen, which we call *RIF2* (*Rap1p-interacting factor 2*). As shown in Table 1, the GAD/Rif2p hybrid encoded by the library plasmid interacts specifically with LexA/Rap1(635–827), but not with LexA alone or an unrelated LexA hybrid (LexA/lamin). Furthermore, when the reading frame between the GAD sequence and the *RIF2* insert was disrupted (creating GAD/rif2fs) no activation of transcription was observed. Thus, the interaction with LexA/Rap1(635–827) is dependent on the GAD/Rif2 fusion protein.

LexA/Rap1(635–827) contains not only the Rap1p silencing domain, but also most of a transcriptional activation domain, which spans amino acids 630–695 (Hardy et al. 1992a). In addition, this hybrid protein has been shown to be a transcriptional activator in cells mutated for either *SIR2*, *SIR3*, *SIR4*, or *RIF1* (Moretti et al. 1994). Therefore, we wanted to determine whether expression of the GAD/Rif2 fusion was able to cause increased activation from other LexA fusion proteins that are themselves weak or cryptic activators. Both LexA/Gcr1(4–419) and LexA/Cib2(15–491) activate transcription from LexA operators to a low level. However, no increase in transcriptional activity of these fusions was observed in the presence of GAD/Rif2 (Table 2), further demonstrating that the interaction of GAD/Rif2 with Rap1p is specific. Sequence analysis of the GAD/Rif2 plasmid revealed that the insert encoded a large open reading frame

Table 1. GAD/Rif2 interacts specifically with LexA/Rap1

	GAD	GAD/Rif2	GAD/rif2fs ^a
LexA/Rap1 (635–827)	7.1	249	6.9
LexA	3.5	3.7	3.6
LexA/lamin	3.7	3.7	3.4

β-Galactosidase activity in CTY10-5D cells determined as described previously (Moretti et al. 1994).

^aGAD/rif2fs contains the same Rif2 insert as GAD/Rif2 but has a frameshift mutation introduced between the *GAL4* activation domain sequences and the *RIF2* sequences.

Table 2. *GAD/Rif2* does not increase the function of other weak activators

	GAD	GAD/Rif2	Fold increase
LexA/Rap1 ^a	7.5	271	36.2
LexA/Gcr1 ^a	39.3	29.2	0.7
LexA/Clb2 ^a	44.6	33.3	0.8

For determination of β -galactosidase activity, see Table 1 footnote.

^aThe LexA fusions used encoded amino acids 635–827 of Rap1p, 4–419 of Gcr1p, and 15–491 of Clb2p.

(see below and Fig. 2A) fused in frame, at amino acid 14, with the *GAL4* activation domain sequences.

Localization of a minimal *Rif2p*-interacting domain within *Rap1p*

Sir3p, Sir4p, and Rif1p interact with a large portion of the carboxyl terminus of Rap1p encompassing part of the putative activation domain and all of the sequences carboxy-terminal to it (Hardy et al. 1992b; Moretti et al. 1994). To determine whether a similar region was required for interaction with Rif2p, we tested a series of LexA/Rap1 fusions with increasing amino-terminal deletions. Proteins of the expected size for all LexA/Rap1 fusions were visible by Western blotting with a LexA-specific antiserum (data not shown). As shown in Figure 1, GAD/Rif2 was able to interact with amino-terminal truncations up to and including LexA/Rap1(679–827) (the numbers in parentheses indicate the Rap1p amino acids present in the hybrid), but failed to interact with hybrids beginning at amino acid 691 or 702 in Rap1p. This pattern is very similar to that of GAD/Sir3(307–978) and GAD/Rif1(1614–1916), but unlike that of GAD/Sir4(1204–1358), which interacts only with the larger LexA/Rap1(635–827) hybrid (Moretti et al. 1994).

To determine the carboxy-terminal boundary of the Rif2p-interacting region of Rap1p we used a series of LexA/Rap1 fusions with a common amino-terminal fusion point, at amino acid 667. In contrast to the results obtained with Sir3p and Rif1p hybrids (Moretti et al. 1994), in which mutation at amino acid 825 or deletion to amino acid 799 of Rap1p severely weakened the interactions with these proteins, the same LexA/Rap1 mutations actually increased the signal obtained with GAD/Rif2 (Fig. 1). The two shorter Rap1p fusions tested, encoding amino acids 667–756 and 667–716, were unable to interact with GAD/Rif2. Thus, the region of Rap1p required for interaction with Rif2p lies between amino acids 679 and 799. In contrast, the Rap1p interaction with Rif1p or Sir3p requires amino acids 679–827, and an even larger region is required for interaction with Sir4p (Moretti et al. 1994).

The results described above indicate that Rif2p can interact with a smaller region of the Rap1p carboxyl terminus than that required by either Sir3p, Sir4p, or Rif1p, suggesting that the Rif2p–Rap1p interaction does not re-

quire these other Rap1-interacting factors. To test this idea more directly, we examined the GAD/Rif2 interaction with LexA/Rap1(647–827) in strains containing mutations in either *SIR3*, *SIR4*, or *RIF1*. We chose this particular LexA/Rap1 hybrid because it still interacts with Sir3p and Rif1p hybrids but does not become a transcriptional activator when *SIR* genes or *RIF1* are mutated (Moretti et al. 1994). As shown in Table 3, mutation of *SIR3* or *SIR4* results in a ~12- and 16-fold increase, respectively, in the apparent interaction between Rap1p and Rif2p. However, mutation of *RIF1* results in a much more dramatic (>100-fold) increase in the Rap1p–Rif2p interaction. These data clearly indicate that the Rap1p–Rif2p interaction is not dependent upon native Sir3p, Sir4p, or Rif1p, and provide further support for the idea that this is a direct interaction. In addition, they raise the possibility that all three proteins (Sir3p, Sir4p, and Rif1p) compete with Rif2p for binding to the Rap1p carboxyl terminus.

Figure 1. GAD/Rif2 interacts with amino acids 679–799 of Rap1p. Two series of LexA/Rap1 fusions with increasing amino- and carboxy-terminal truncations were assayed for interaction with GAD/Rif2 in CTY10-5D cells. β -Galactosidase activities were determined from liquid cultures as described previously (Moretti et al. 1994). Each fusion was also tested with GAD alone as a control.

Table 3. Deletion of genes encoding known *Rap1p*-interacting factors increases the interaction of *LexA/Rap1* with *GAD/Rif2*

	GAD	GAD/Rif2
Wild type	3.6	20.9
<i>sir3::HIS3</i>	5.7	259
<i>sir4::HIS3</i>	5.9	332
<i>rif1::HIS3</i>	4.9	2244

For determination of β -galactosidase activity, see Table 1 footnote. In all cases, the DNA-binding domain hybrid is LexA/Rap1 (647–827). The wild-type strain is CTY10-5D, and the mutants were derived from this strain by gene disruption (Moretti et al. 1994).

Rif2p interacts with *Rif1p*

An alternative explanation of the results described above (Table 3) is that *Rif2p* normally interacts with the *Sir* proteins or with *Rif1p* (in addition to *Rap1p*), and that the binding of *GAD/Rif2* to these proteins somehow prevents or weakens a functional interaction with LexA/Rap1 in the two-hybrid system. In this case, mutation of the *SIR* or *RIF1* genes might leave more *GAD/Rif2* hybrid free to interact productively with LexA/Rap1. To explore this possibility we created a LexA/Rif2 fusion protein containing amino acids 2–395 of *Rif2p*. This fusion protein did not activate transcription on its own and no significant increase in β -galactosidase activity was observed upon coexpression of either *GAD/Sir3* or *GAD/Sir4*, suggesting that these proteins do not interact with *Rif2p* (Table 4). However, a *GAD/Rif1* fusion was able to interact with LexA/Rif2(2–395), giving a signal >20-fold over the background with *GAD* alone (Table 4). Thus, it appears that *Rap1p*, *Rif1p*, and *Rif2p* are all able to interact with each other. In light of these results, the simplest interpretation of the effect of *SIR* mutations on the *Rif2p*–*Rap1p* interaction (Table 3) would be that *Sir3p* and *Sir4p* compete for binding to the *Rap1p* carboxyl terminus with *Rif2*. In the case of *Rif1p*, because of the observed interaction with *Rif2p*, we favor the idea that the *RIF1* mutation improves the *Rap1p*–*Rif2* interaction by freeing *GAD/Rif2* to interact more productively with the LexA/Rap1 hybrid. However, we cannot rule out the possibility that *Rif1p* also competes with *Rif2p* for binding to *Rap1p*.

Rif2p and its interaction with *Rif1p* and *Rap1p*

Analysis of the amino-acid sequence of *Rif2p* (Fig. 2A), reveals that it encodes a protein of 395 amino acids with a predicted molecular mass of 46 kD. (The *RIF2* gene is on chromosome XII-R, hypothetical protein YLR453c, GenBank accession no. U22382.) Western analysis of epitope-tagged *Rif2p*, expressed from its own promoter, revealed a protein of the expected size (data not shown). This protein has no significant homology to other known sequences and no convincing structural motifs. The only notable features of the amino acid sequence of

Rif2p are the presence of a lysine-rich region (21.3% lysine) spanning amino acids 15–75 and a second basic region at the carboxyl terminus (17.1% lysine and 14.3% arginine over 35 amino acids).

To determine which region of *Rif2p* was responsible for the interaction with *Rap1p* and *Rif1p*, we created a series of LexA/*Rif2* fusions and tested their interactions with *GAD/Rap1* and *GAD/Rif1*. As shown in Figure 2B, truncation of the carboxyl terminus of *Rif2p* to amino acid 388 did not affect the interaction with *Rap1p* or *Rif1p*, but further truncation (to amino acid 332) abolished the interaction with both proteins. Both *GAD/Rap1* and *GAD/Rif1* were able to interact with *Rif2p* fusions starting at amino acid 2 or 18. However, deletion of the first 49 amino acids of *Rif2p* abolished the interaction with *Rap1p* (Fig. 2B). In contrast, the LexA/*Rif2*(50–395) hybrid interacted strongly with *GAD/Rif1*, in fact giving more than twice as many β -galactosidase units as the LexA/*Rif2*(2–395) hybrid, which interacts with both *Rap1p* and *Rif1p*. A further deletion of 40 amino-terminal residues, giving LexA/*Rif2*(90–395), resulted in a hybrid unable to interact with either the *Rap1p* or the *Rif1p* hybrid. Thus, *Rap1p* and *Rif1p* interact with largely overlapping regions of *Rif2p*, although there does appear to be a difference between the regions of *Rif2p* required for interaction with these two proteins. The fact that *GAD/Rif1* interacts with LexA/*Rif2*(50–395), whereas *GAD/Rap1* is unable to, suggests that the *Rif1p*–*Rif2p* interaction is independent of *Rap1p*.

We further analyzed these interactions using cells in which either *RIF1* or *RIF2* had been deleted. A *RIF2* disruption was created by replacing the sequence encoding amino acids 18–389 of *Rif2p* with the *HIS3* gene. Cells lacking the *RIF2* gene were viable and appeared to grow normally. Deletion of either *RIF1* or *RIF2* did not result in loss of any of the interactions described above between *Rap1p*, *Rif1p*, and *Rif2p*, suggesting that none of these proteins is bridging the interactions between any other pair (data not shown).

RIF2 mutation affects silencing at both HMR and telomeres

The known *Rap1p*-interacting proteins, *Sir3p* and *Sir4p* on the one hand, and *Rif1p* on the other, clearly play different roles in transcriptional silencing. Null mutations of either *SIR3* or *SIR4* completely abolish silencing at both telomeres and *HM* loci (Ivy et al. 1986; Rine and

Table 4. *Rif2p* interacts with *Rif1p*

	GAD	GAD/Sir3	GAD/Sir4	GAD/Rif1
LexA	5.1	4.8	5.3	6.0
LexA/ <i>Rif2</i>	4.5	9.1	7.1	91.7

For determination of β -galactosidase activity, see Table 1 footnote. The *GAD* fusions are as described in Moretti et al. (1994). The LexA/*Rif2* fusion encodes amino acids 2–395 of *Rif2p* and was generated by PCR (see Materials and Methods).

Figure 2. Rif2p residues required for the interactions with Rap1p and Rif1p. (A) The predicted amino-acid sequence of Rif2p. The position of the original GAD fusion isolated is indicated by an arrow. A lysine-rich region near the amino terminus and a basic carboxy-terminal stretch are underlined. (B) A series of LexA/Rif2 fusions were created to identify the region of the protein required for interaction with Rap1p and Rif1p. β -Galactosidase activities were determined as in Fig. 1.

Herskowitz 1987; Aparicio et al. 1991). *RIF1* mutations, however, actually improve silencing at telomeres while weakening repression at *HMR* loci containing a mutated *HMR-E* silencer (Hardy et al. 1992b; Kyrion et al. 1993). It is thought, therefore, that Sir3p and Sir4p are absolutely required for silencing, consistent with the observation that these proteins appear to be structural components of silent chromatin (Hecht et al. 1995, 1996). Rif1p appears to play a regulatory role in silencing by affecting the balance between telomeric and *HM* locus silencing, a conclusion that is supported by studies of the unusual properties of *rap1^s* mutations, which create a defect in the Rap1p–Rif1p interaction (Buck and Shore 1995).

We therefore examined the effect of *RIF2* mutation on silencing at *HMR* and at a genetically marked telomere. To examine silencing at *HMR*, we used a sensitive reporter gene (*TRP1*) and an *HMR-E* silencer containing a mutation of the origin recognition complex (ORC) binding site (*HMR Δ A::TRP1*) (Sussel and Shore 1991). *rap1^s* and *rif1* mutations result in derepression at *HMR* only in the context of this weakened silencer (Sussel and Shore 1991; Hardy et al. 1992b). In an otherwise wild-type strain background, the *hmr Δ A::TRP1* reporter is strongly repressed, as indicated by the absence of growth on medium lacking tryptophan (SC–Trp, Fig. 3A). Little or no effect of a *RIF2* mutation alone is observed, whereas deletion of *RIF1* results in a clear increase in the expression of the *TRP1* reporter gene at *HMR Δ A*, as reported previously (Hardy et al. 1992b). Interestingly, despite the absence of a clear effect of the *rif2* mutation, introduction of the *rif2* disruption into a *rif1 HMR Δ A::TRP1* strain led to a significant further derepression of the reporter gene, as indicated by the ability of essentially all of the cells to grow in the absence of tryptophan (Fig. 3A). Thus, although a *rif2* mutation alone has little or no effect on *HMR*, in combination with a *rif1* mutation it has a synergistic effect on silencing at this locus.

To examine the effects of *RIF* mutants on telomere

Figure 3. Deletion of *RIF2* affects silencing. (A) Strains were created with an *hmr Δ A::TRP1* reporter and gene disruptions of either *RIF1*, *RIF2*, or both together. YDW76 (*RIF1 RIF2*), YDW123 (*rif1::URA3 RIF2*), YDW77 (*RIF1 rif2::HIS3*), and YDW124 (*rif1::URA3 rif2::HIS3*) were assayed for *TRP1* expression (see Materials and Methods). The relevant genotypes are shown on the left. (B) Telomeric silencing in strains (YDW84, YDW85, YDW86, and YDW87) with a telomeric *ADE2* reporter was assayed by colony color. Repression of the telomeric *ADE2* gene results in red sectors or colonies and *ADE2* expression results in white colonies. A single white colony of each strain was grown overnight in YPD and 200–300 cells were plated onto YPD plates. Cells were photographed after 2–3 days at 30°C and 2 days at 4°C.

position effect (TPE), we assayed the expression of a telomeric *ADE2* reporter gene by observing colony color. Expression of *ADE2* results in white colonies, whereas in cells that do not express the *ADE2* gene red colonies are observed on plates containing minimal adenine. Colonies containing red and white sectors represent a mixed population of cells in which the *ADE2* gene is repressed as well as expressed. For each strain, a single white colony was picked and grown overnight in rich medium, after which 200–300 cells were spread onto YPD plates and the colony color observed. In wild-type cells very few sectorized or red colonies were observed (<1%), suggesting that the *ADE2* gene was expressed in most cells, and that this derepressed state was relatively stable (Fig. 3B). Deletion of *RIF2* resulted in a small but reproducible increase in the number of sectorized colonies to ~11% (Fig. 3B). Mutation of *RIF1* has been shown previously to increase TPE (Kyrion et al. 1993), and as shown in Figure 3B, this effect is larger than that seen in *rif2* mutants (~34% sectorized colonies). In cells lacking both Rif1p and Rif2p, a large proportion of the colonies was either completely red or contained red sectors (>50%; Fig. 3B). Thus, although mutation of *RIF2* increased telomeric silencing only slightly, it had a synergistic effect when combined with a *rif1* mutation.

In summary, the *rif1* phenotype is stronger than that of *rif2* in both *HMR* silencing and TPE assays. Furthermore, despite the relatively weak effect of a *rif2* mutation in either assay, it has a strong synergistic effect together with a *rif1* mutation on the balance between telomeric and *HMR* silencing, significantly strengthening the former and weakening the latter.

RIF1 and *RIF2* mutations have a synergistic effect on telomere elongation and chromosome loss

One possible explanation for the strong effect of the *rif2* mutation on silencing only when present together with a *rif1* mutation is that *rif2* significantly exacerbates the telomere elongation effect of *rif1* mutations (Hardy et al. 1992b). Extreme telomere elongation (caused by *rap1^s* mutations) can increase TPE in wild-type cells (Kyrion et al. 1993) while exerting an opposite effect on silencing at *HMR* (Buck and Shore 1995). We therefore examined the length of the telomeric repeats in *rif2* and *rif1 rif2* double mutant cells. As shown in Figure 4, deletion of *RIF2* alone results in an increase of ~100 bp in the average length of Y'-containing telomeres (which yield the broad lower band in the *XhoI* digest shown). For comparison, a *RIF1* deletion resulted in a somewhat greater increase in average length and heterogeneity of telomeres, as shown previously (Hardy et al. 1992b). In DNA from cells lacking both *RIF1* and *RIF2*, the length of the telomeric repeat sequences is increased by at least 600 bp, and up to 2.5 kb (Fig. 4), which is considerably greater than the increase for either single *RIF* mutant. Thus, Rif1p and Rif2p probably perform different functions in telomere length regulation.

The fact that the telomere elongation observed in severe *rap1^s* mutants (which are unable to interact with

Figure 4. Increased telomere length in *RIF*-mutant cells. DNA was isolated from wild-type, *rif1*, *rif2*, and *rif1 rif2* cells (YDW76, YDW123, YDW 77, and YDW124) and was digested with *XhoI* and hybridized to reveal telomere repeat sequences, as described in Materials and Methods. In wild-type cells, this digest releases a broad band of ~1.2 kb containing the telomeric repeat sequences from the majority of telomeres (see Zakian 1995a). The relevant genotypes are indicated above the lanes.

Rif1p in two-hybrid assays) is similar to that seen in *rif1* cells suggests that this allele of *RAP1* retains some ability to regulate telomere length. Consistent with this, we found that Rif2p is able to interact with *rap1^s* mutants in the two-hybrid assay (data not shown). However, this interaction was reduced to ~50% of that seen with wild-type Rap1p fusions, indicating that the *rap1^s* mutations do not specifically affect the Rap1p–Rif1p interaction, but instead may be having a more general effect on the carboxy-terminal region of Rap1p. This reduced *rap1^s*–Rif2p interaction may explain why *rap1^s rif1* double mutants have a more severe telomere elongation phenotype than either single mutant (L. Sussel, S. Buck, and D. Shore, unpubl.). An additional possibility is that some Rif1 protein is recruited to telomeres in *rap1^s* cells via the Rif1p–Rif2p interaction.

As mentioned above, previous work has shown that a large carboxy-terminal truncation of Rap1p (in particular the *rap1^s* allele *rap1-17*, in which amino acids 663–827 are missing) results in a dramatic increase in telomere length (Kyrion et al. 1992). The protein produced by the

Figure 5. Telomere length in *rap1-17* cells and in cells lacking both *RIF1* and *RIF2*. DNA was isolated and treated as in Fig. 4. The genotypes of the cells are shown at the top. (+) Wild type; (–) mutant for *RIF1* or *RIF2* as shown. The *RAP1* alleles were either wild-type or *rap1-17*, which expresses a Rap1 protein lacking the carboxy-terminal 164 amino acids. The strains used in this analysis (YDW126–YDW131) were generated by sporulation and dissection of the diploid YDW125, which is heterozygous for the *rap1-17* allele and disruptions of both *RIF1* and *RIF2*.

rap1-17 allele would be unable to interact with either Rif1p or Rif2p, as judged by our two-hybrid analyses (Hardy et al. 1992b; Fig. 1). It was therefore of interest to determine whether the changes in telomere length in a *rif1 rif2* double mutant are comparable to those seen in a *rap1-17* strain. To compare these strains more easily we crossed a *rap1-17* strain with a strain mutated for both *RIF1* and *RIF2* to create a diploid that was heterozygous for each mutation. This diploid was sporulated to obtain strains with combinations of these three mutations. As shown in Figure 5, a similar increase in telomere length and heterogeneity is observed when Rap1p is truncated or when both *RIF1* and *RIF2* are mutated. Additionally, no further increase in telomere length was observed in cells carrying the *rap1-17* allele in combination with mutations in *RIF1* and *RIF2* (Fig. 5). These results indicate that the Rap1p carboxyl terminus and the Rif1/Rif2 protein pair act in the same pathway to regulate telomere length. In other words, the function of the Rap1p carboxyl terminus can be explained, at least in principle, by its ability to recruit Rif1p and Rif2p to the telomere.

To further address the functional relationships between Rif1p, Rif2p, and the Rap1p carboxyl terminus, we measured chromosome loss rates in *RIF* single mutants and the *rif1 rif2* double mutant. Previous studies have shown that *rap1^t* mutants display elevated chromosome loss rates, perhaps as a result of impaired telomere function (Kyrion et al. 1992). As shown in Table 5, we found that chromosome III loss rates in *rif1* and *rif2* mutants were elevated by ~7.5- and 3.5-fold, respectively, compared with wild-type. However, just as for silencing and telomere length measurements, the *rif1 rif2* double mutant displayed a synergistic effect, giving a chromosome III loss rate >30-fold greater than wild type. This relative increase is comparable to that determined for the most severe *rap1^t* mutant (*rap1-17*) using a different chromosome loss assay (Kyrion et al. 1992).

Additionally, cells mutated for both *RIF1* and *RIF2* have a significantly longer doubling time than wild-type cells (Table 5). In contrast, mutation of either *RIF* gene alone does not significantly alter the growth rate, providing further evidence for synergistic action of Rif1p and Rif2p.

Overexpression of Rif1p and Rif2p reduces telomere length and reverses the effect of Rap1p carboxy-terminal overexpression

It has been demonstrated previously that overexpression of the carboxyl terminus of Rap1p, in the absence of its DNA-binding domain, increases telomere length (Conrad et al. 1990; Hardy 1991). The likely explanation of this effect is that the overexpressed carboxyl terminus of Rap1p interacts with proteins involved in telomere length regulation, titrating them away from the telomeres and thus causing telomere elongation. We reasoned that if Rif1p and Rif2p were responsible for telomere length regulation, and in particular acted as negative regulators of telomere elongation, overexpression of these proteins might shorten telomeres in wild-type cells and restore normal telomere length to cells in which the carboxyl terminus of Rap1p is overexpressed. As shown in Figure 6 (lanes 2,3), the telomeric repeats in

Table 5. Phenotypes of cells lacking Rif1p and Rif2p

Genotype	Telomere length increase ^b (bp)	Doubling time ^c (min)	Chromosome loss rate ^d (per generation)
Wild type	N.A.	90	4.3×10^{-7}
<i>rif1</i>	200–600	92	3.2×10^{-6}
<i>rif2</i>	100	90	1.5×10^{-6}
<i>rif1 rif2</i>	600–2500	108	1.5×10^{-5}

^aAll strains are isogenic to W303 except for the *RIF* mutations indicated.

^bDerived from Figs. 4 and 5.

^cGrowth rates were measured for exponentially growing cells in rich medium (YPD) at 30°C.

^dBased on determination of rate of loss of chromosome III (see Materials and Methods for details).

Figure 6. Effects of overexpression of Rif1p and Rif2p on telomere length. Wild-type W303-1B cells (*MATa*) were transformed with plasmids containing the genes encoding the proteins indicated at the top. Rif1p, Rif2p, Sir3p, and Sir4p were expressed as full-length proteins from their own promoters, present on either multicopy (2 μ -based) or low-copy (CEN/ARS-based) plasmids. (ctrl) The presence of a control plasmid lacking an insert. (Rap1 C-ter) The presence (+) of a plasmid encoding amino acids 630–827 of Rap1p fused to LexA. This fusion, which lacks the Rap1p DNA-binding domain, is expressed from the strong *ADH1* promoter on a multicopy (2 μ) plasmid. DNA was prepared and telomere repeat lengths analyzed as in Fig. 4.

wild-type cells carrying either full-length *RIF2* or *RIF1* on a multicopy plasmid were reduced in length. No effect of a multicopy *SIR4*-containing plasmid was observed (Fig. 6, lane 4). In contrast, cells expressing LexA/Rap1(630–827) from the strong *ADH1* promoter (on a multicopy plasmid) had slightly elongated telomeres (Fig. 6, lane 5), consistent with previous studies (Conrad et al. 1990; Hardy 1991). Strikingly, overexpression of either *RIF1* or *RIF2*, but not *SIR4*, resulted in a reduction in telomere length in the presence of LexA/Rap1(630–827) (Fig. 6, lanes 6–9). The *RIF1* high-copy plasmid sig-

nificantly reduced telomere lengths to nearly wild-type levels, whereas *RIF2* had a small effect.

We also examined the effect on telomere length of the addition of single extra copies of the *RIF* and *SIR* genes in the presence of carboxy-terminal overexpression of Rap1p. The addition of *RIF1* on a centromere-based plasmid was able to partially suppress the effect of the LexA/Rap1(630–827) plasmid. Strikingly, when *RIF1* and *RIF2* were present together on centromeric plasmids in these cells, a reduction in telomere length and heterogeneity almost to that of wild-type cells was observed (Fig. 4, lanes 10–13). No effect of either *SIR3* or *SIR4* alone was observed, although some suppression of telomere elongation was observed with both together (Fig. 6, lanes 14–16). Taken together, these results suggest that the levels of Rif1p and Rif2p can have a direct effect on the regulation of telomere length, and provide further indication that these two proteins play a critical role in this process.

Discussion

Several lines of evidence indicate that Rap1p, which binds to multiple high-affinity sites within the poly (C_{1–3}A) tracts at telomeres, plays a critical yet complex role in telomere length regulation. Temperature-sensitive *RAP1* mutants, grown under semi-permissive conditions, have shorter telomeres (Conrad et al. 1990; Lustig et al. 1990), suggesting that one function of Rap1p may be simply to protect the telomere from degradation by exonucleases. However, this is unlikely to be the sole function of Rap1p in telomere length regulation. Lustig and colleagues have shown that carboxy-terminal truncation mutations of *RAP1*, which retain the DNA-binding domain and are viable, exhibit an extreme telomere-elongation phenotype (Kyrion et al. 1992). It is presumed, therefore, that this nonessential domain of Rap1p, which is also required for telomere position effect and *HM* locus silencing (Kyrion et al. 1993; Moretti et al. 1994), negatively regulates telomere elongation. This might occur, for example, by direct inhibition of telomerase or by controlling the access of telomerase to the chromosome end. Whatever the mechanism of telomere length regulation by Rap1p, it is likely to work through interactions with other proteins because overexpression of the Rap1p carboxyl terminus, in the absence of its normal DNA-binding domain, results in telomere elongation (Conrad et al. 1990; Hardy 1991). These data can be most easily explained by the existence of titratable factors that interact with the Rap1p carboxyl terminus to mediate telomere length regulation. The first candidate for such a factor to be identified was Rif1p (Rap1p-interacting factor 1), which was isolated by a two-hybrid screen using the carboxy-terminal 175 amino acids of Rap1p (Hardy et al. 1992b). However, the effect on telomere length of mutating *RIF1* is relatively small by comparison with the Rap1p truncation mutants.

Rif1p and Rif2p are required to mediate the telomere length regulation function of Rap1p

Here we have described a new Rap1p-interacting factor

(Rif2p) with properties very similar to those of Rif1p. Both of these proteins interact with the Rap1p carboxyl terminus in two-hybrid assays, and mutation of either protein results in moderate telomere elongation and an increase in TPE. Significantly, cells lacking both of these proteins have extremely elongated telomeres that are indistinguishable from those in cells containing a *rap1^t* mutation, which removes the Rap1p carboxyl terminus. The simplest interpretation of these observations is that the Rap1p carboxyl terminus recruits both Rif1p and Rif2p to telomeres where they are required to regulate telomere elongation. One prediction of this model, which we are at present trying to test, is that Rif proteins are localized at telomeres in vivo. Our results do not indicate whether or not Rif1p and Rif2p are sufficient to mediate the telomere length regulation function of Rap1p, but they do indicate that both are necessary. If other Rifs are involved in this regulatory function, their action must be dependent upon either *RIF1*, *RIF2*, or both. Finally, the fact that neither *RIF1* nor *RIF2* mutation exacerbates the telomere elongation phenotype of the *rap1-17* allele suggests that the function of these two proteins in telomere length regulation requires the Rap1p carboxyl terminus.

Because Rif1p and Rif2p can interact with each other in the two-hybrid system, these two proteins may act as a complex that is recruited to the telomere. If this is the case, it might seem odd that the two proteins have distinct functions, as indicated by the synergistic effect of a *rif1 rif2* double mutation on telomere length. One possible explanation for these observations is that, once recruited to the telomere, Rif1p and Rif2p interact with different proteins, and thus genetically and biochemically define two regulatory pathways for telomere length regulation. Alternatively, Rif1p and Rif2p might have a common target. In this case each protein would by itself provide partial regulation by interacting independently with this target. More information concerning the mechanism of action of both proteins will be required before these models can be distinguished. Another interesting possibility raised by the interaction of Rif1p with Rif2p is that Rif1p at one telomere may interact with Rif2p at another. Thus, the Rif proteins may play a role in the clustering of telomeres into groups that has been observed by immunolocalization of telomere-associated proteins and in situ hybridization with telomere-specific probes (Klein et al. 1992; Palladino et al. 1993; Gotta et al. 1996). Finally, we should point out that it is unclear at present what, if any, role Rif1 and Rif2 might play in a novel mechanism of telomere length control, called "telomeric rapid deletion" (Li and Lustig 1996), which can reduce extremely elongated telomeres to wild-type lengths in what appears to be a single-division event.

Rif and Sir proteins mediate different functions of Rap1p at telomeres

Because the initial characterization of *RIF1* revealed a complex set of effects on telomeric and *HM* locus silencing as well as telomere length control (Hardy et al.

1992b; Kyrion et al. 1993), the precise function of this protein was unclear. The identification and characterization of Rif2p reported here greatly clarifies this picture by providing evidence for a specialized mechanism of telomere length regulation by Rap1p, separate from its silencing function. The fact that Rif2p is required together with Rif1p for telomere length control, and that these two proteins interact with each other in two-hybrid assays, supports the idea that their primary role is to negatively regulate telomere elongation. Several lines of evidence indicate that the changes in silencing brought about by *RIF* mutations are likely to be secondary consequences of their effects on telomere length and structure. To begin with, telomere elongation in wild-type cells is sufficient to cause increased TPE (Kyrion et al. 1993) and decreased silencing at *HMR* (Buck and Shore 1995). In principle, therefore, the effects of *RIF* mutations on silencing could be an indirect consequence of their effects on telomere structure. Second, Rif proteins, unlike the Sir proteins, are not required for silencing, but rather affect the balance between telomeric and *HMR* locus silencing mediated by competition for Sir proteins (Buck and Shore 1995). As such, the Rif proteins can be viewed as regulators of silencing, but are not required components of silent chromatin, like Sir3p and Sir4p (Hecht et al. 1995, 1996). Finally, it is important to keep in mind that the effects of *SIR3* and *SIR4* mutations on telomeres are exactly opposite those of *RIF1* and *RIF2* mutations. In the *SIR* mutants TPE is abolished (Aparicio et al. 1991) and telomere tracts become slightly shorter (Palladino et al. 1993), whereas *RIF* mutations improve TPE (Fig. 3B; Kyrion et al. 1993) and increase telomere length (Fig. 4; Hardy et al. 1992b).

In a separate report (Marcand et al. 1997) we present evidence that telomere length is regulated by a negative feedback mechanism that can sense the precise number of Rap1 carboxyl termini at the chromosome end. The data presented here suggest that the Rif1p/Rif2p complex, bound to Rap1p at telomeres, mediates this length-sensing and regulation function of Rap1p, which is itself antagonized by Rap1p–Sir interactions. Putting these and other data together, we propose a model in which Rap1p–Sir and Rap1p–Rif complexes are naturally partitioned to opposite ends of the telomeric $C_{1-3}A$ repeats (see Fig. 7). We imagine that Rap1p–Sir interactions are favored at the proximal end of the telosome (Wright et al. 1992) (the telosome/nucleosome junction) by cooperative interactions between Sir proteins themselves (Moretti et al. 1994) and between Sir proteins and histones H3 and H4 (Hecht et al. 1995). Because TPE is likely to result from a continuous spreading of Sir complexes along the nucleosome fiber, it follows that the Rap1p molecules at the junction between the telosome and nucleosomal DNA would be most critical for establishing silencing. On the other hand, the Rif1p/Rif2p complex is likely to act at the telomere end, and we speculate that Rif protein assembly at the distal end of the telosome might be promoted by interactions with telomere end-binding proteins. We note that a tendency of Rif complexes to assemble at the distal end of the telomere

Figure 7. A speculative model for the arrangement of Rif and Sir protein complexes at telomeres. Partitioning of the telosome (Wright et al. 1992) into centromere-proximal Sir and telomere-proximal Rif domains is diagrammed, together with the effects of increased (A) and decreased (B) expression of Rif proteins. We propose that the extent of the Rap1p–Rif protein complex may play a direct role in regulating telomere length. In the case of Rif protein overexpression we imagine that Rif complex spreads from the telomere end and results in a gradual shortening of the C₁₋₃A tract until a new stable equilibrium value is reached in which Rif complex addition and telomere shortening are in balance. Under conditions of decreased Rif expression we propose a loss of Rif complex at the telosome and a corresponding increase in Sir complex (which results in improved TPE). This reduction of Rif complex results in telomere elongation, which, depending upon the severity of the defect, may lead to a longer, stable telomere length (*rif1* or *rif2* single mutants) or essentially unregulated telomere elongation (*rif1 rif2* double mutant).

might help to explain recent observations in *Kluyveromyces lactis* that suggest that Rap1p-binding sites nearest to the telomere end play a critical role in length regulation (Krauskopf and Blackburn 1996). Nevertheless, we would emphasize that this model is speculative, and that the available data do not rule out a model in which Sir and Rif complexes are interdigitated along the telosome. It seems likely that a direct biochemical and structural characterization of the telosome will be required to distinguish between these two models.

Whatever the precise arrangement of Sir and Rif complexes along the telosome, the critical parameter controlling telomere length regulation would appear to be the amount of Rif complex assembled there. The shortening of telomeres caused by the overexpression of Rif proteins (Fig. 6) would thus result from an extension of the Rif complex along the telosome that results in telomere shortening, possibly by inhibition of telomerase (Fig. 7A). Conversely, mutation of one of the *RIF* genes may result in a decrease in the amount of the telomere bound by Rif protein, allowing telomerase activation and telomere elongation (Fig. 7B). The removal of Sir3p or Sir4p by mutation would allow free access of the Rif1/Rif2 complex to the telosome, which would cause increased telomerase inhibition and result in telomere shortening, which is precisely what has been observed (Palladino et al. 1993). Our model for telomere length regulation (Marcand et al. 1997) predicts that the extent of telomere shortening in *SIR3* or *SIR4* mutants (~50 bp) would reflect the amount of C₁₋₃A tract occupied by Rap1p–Sir complexes (thus excluding Rif1/Rif2) in wild-type cells. Assuming that Rap1p binding sites occur approximately every 18 bp along the telomere, this would translate to about three Rap1p–Sir complexes.

How do Rif proteins regulate telomere length?

Our data can be most simply explained by proposing that Rif proteins are recruited to telomeres by Rap1p where they either block telomere elongation directly or promote degradation of the ends. To explain how this regulatory mechanism can be highly sensitive to the number of Rap1p molecules (and ultimately Rif complexes) bound at an end, we would propose that either a stochastic process controls events at the end through interactions with a Rap1p/Rif complex, as suggested previously for the yeast *K. lactis* (McEachern and Blackburn 1995), or the Rap1p/Rif complex creates a length-sensitive switch to control the access of factors to the telomere end or their activity once bound there (Marcand et al. 1997). One obvious candidate for Rif action is the telomerase enzyme itself, which has been detected recently in vitro (Cohn and Blackburn 1995; Lin and Zakian 1995), and whose RNA template is now known (Singer and Gottschling 1994). Another candidate for Rif action is the product of the *PIF1* gene, a helicase that inhibits telomere elongation (Schulz and Zakian 1994). One could imagine that Rif proteins target this enzyme to telomeres. However, genetic epistasis tests suggest that Pif1p acts in a different pathway from the Rap1p carboxyl terminus (Schulz and Zakian 1994). Alternatively, the Rif proteins may interact with one or more recently identified telomere end-binding proteins to regulate telomerase. The Est1 (Virta-Pearlman et al. 1996) and Cdc13(Est4) (Lin and Zakian 1996; Nugent et al. 1996) proteins both bind to single-strand TG₁₋₃ telomeric sequences and appear by genetic criteria to be essential regulators of telomerase activity (Lundblad and Szostak 1989; Garvik et al. 1995; Nugent et al. 1996). A newly

identified Cdc13p-interacting protein with similar functions, Stn1p, is another potential Rif target (Grandin et al. 1997). Given our present limited understanding of telomere replication, it seems likely that there are other possible mechanisms of Rif1 action. Identifying factors other than Rap1p that interact with the Rif proteins, and studying genetic and biochemical interactions between the *RIF* genes and known telomere replication factors, should provide important clues to understanding how the Rif proteins work.

Materials and methods

Plasmids

All LexA fusions were expressed from pBTM116 (2 μ m origin, *TRP1*, *pADH1-lexA*; a gift of P. Bartel and S. Fields, State University of New York, Stony Brook). The LexA/Rap1 fusions are as described previously (Moretti et al. 1994), except for LexA/Rap1(679–756), which was created by PCR. The GAD-fusion library screened with LexA/Rap1 was created in pGAD3 (Chien et al. 1991) and was a generous gift of P. Bartel and S. Fields. GAD/*rif2fs* was created by digesting GAD/Rif2 with *Mlu*I, end-filling with Klenow, and religating. LexA/Rif2 fusions were generated by PCR, except for those encoding amino acids 18–389 and 18–298, which were created by subcloning fragments from GAD/Rif2 into pBS (Stratagene), then into pBTM116. GAD/Rap1 encoded amino acids 478–827 and was isolated by screening a two-hybrid library with LexA/Rif2(2–395). The Gbd/Rap1

fusions, GAD/Rif1, GAD/Sir3, and GAD/Sir4, are as described previously (Hardy et al. 1992a; Moretti et al. 1994). Wild-type *RIF1*, *RIF2*, *SIR3*, and *SIR4* were expressed from their own promoters, and were present on CEN/ARS- or 2- μ -based pRS vectors (Sikorski and Hieter 1989). The full-length *RIF2* gene, including 495 bp of sequence 5' to the initiation codon and 120 bp 3' to the translational stop, was isolated by PCR and cloned into pBS. The *RIF2* deletion/insertion mutation was created in pBS by replacing the sequences encoding amino acids 18–389 with the *HIS3* gene. *RIF1*, *SIR3*, and *SIR4* disruption mutations have been described previously (Hardy et al. 1992b; Moretti et al. 1994). Sequences of oligonucleotides used in PCR-based cloning steps and details of all plasmid constructs are available upon request.

Yeast strains

The yeast strains used in this study are all derivatives of either W303 (Thomas and Rothstein 1989) or the two-hybrid assay strain CTY10-5D, and are listed in Table 6.

Yeast manipulations

Growth and manipulation of yeast strains was carried out using standard procedures (Rose et al. 1990). Screening of the GAD-fusion library with LexA/Rap1 was carried out in CTY10-5D, as described previously (Moretti et al. 1994). Spot assays were performed by spotting 5 μ l aliquots of 10-fold serial dilutions of a saturated overnight culture onto plates containing the appropriate selective media. Plates were photographed after 2–3 days.

Table 6. Yeast strains used in this study

W303-1A	<i>HMLα MATα HMRα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1</i>
W303-1B	<i>HMLα MATα HMRα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1</i>
CTY10-5D	<i>MATα ade2-1 trp1-901 leu2-3,112 his3-200 gal4 gal80 URA3::LexA op-lacZ</i>
PM101	CTY10-5D <i>sir3::HIS3</i>
PM102	CTY10-5D <i>sir4::HIS3</i>
PM103	CTY10-5D <i>rif1::HIS3</i>
AJL394-1a	W303-1A <i>rap1-17 telVII::ADE2/URA3</i>
YLS607	W303-1B <i>hmrΔA::ADE2 rif1::URA3</i>
YDW62	CTY10-5D <i>rif2::HIS3</i>
YDW76	W303-1B <i>hmrΔA::TRP1 telVII::URA3</i>
YDW77	YDW76 <i>rif2::HIS3</i>
YDW123	YDW76 <i>rif1::URA3</i>
YDW124	YDW76 <i>rif1::URA3 rif2::HIS3</i>
YDW80	W303 <i>HMLα MATα hmrΔA::ADE2</i> <i>HMLα MATα hmrΔA::ADE2</i>
YDW81	YDW80 <i>rif1::URA3/rif1::URA3</i>
YDW82	YDW80 <i>rif2::HIS3/rif2::HIS3</i>
YDW83	YDW80 <i>rif1::URA3/rif1::URA3 rif2::HIS3/rif2::HIS3</i>
YDW84	W303-1A <i>hmrΔA::TRP1 telVII::ADE2/URA3</i>
YDW85	YDW84 <i>rif1::URA3</i>
YDW86	YDW84 <i>rif2::HIS3</i>
YDW87	YDW84 <i>rif1::URA3 rif2::HIS3</i>
YDW125	W303 <i>HMLα MATα hmrΔA::TRP1 rap1-17 rif1::URA3 rif2::HIS3</i> <i>HMLα MATα HMRα RAP1 RIF1 RIF2</i>
YDW126	W303-1A <i>hmrΔA::TRP1</i>
YDW127	W303-1A <i>rap1-17</i>
YDW128	YDW127 <i>rif1::URA3</i>
YDW129	YDW127 <i>rif2::HIS3</i>
YDW130	YDW127 <i>rif1::URA3 rif2::HIS3</i>
YDW131	YDW126 <i>rif1::URA3 rif2::HIS3</i>

β -galactosidase assays were carried out as described previously [Moretti et al. 1994].

Chromosome loss assays

Determination of the rate of loss of chromosome III was carried out as described previously [Chi and Shore 1995]. Briefly, single colonies were resuspended in 1 ml of YPD and sonicated. Dilutions were plated onto YPD medium to assess the number of viable cells. Half of each colony was then incubated with a 100-fold excess of a *MATa* tester strain for 8 hr at 30°C. Cells were then spread onto SD plates to select for colonies that arose from a mating event. The chromosome rate loss was derived according to the following formula: $(0.4343 \times F)/(\log N - \log N_0)$, where F = the frequency of mating events, N = the number of cells in the colony and N_0 = the number of cells from which the colony arose. For this analysis, we used diploid strains that lacked any mating-type information at *HMR*.

DNA sequencing

All sequencing was carried out by use of the dideoxy chain termination method, using Sequenase (Amersham).

Telomere blots

DNA was isolated from overnight yeast cultures and 1 μ g was digested with *Xho*I for 4 hr. DNA was electrophoresed through 0.8% agarose and transferred to nylon membranes (Hybond). Membranes were hybridized at 50°C in 6 $\times$ SSC, 5 $\times$ Denhardt's solution, with a poly[d(G-T)] probe labeled by random priming. Membranes were washed twice in 2 $\times$ SSC at 55°C for 45 min and autoradiographed with Kodak X-AR5 film.

Acknowledgments

We are grateful to A. Lustig for providing strains and to P. Bartel and S. Fields for generously providing the pGAD3 library. We would also like to thank H. Laman, L. Pemberton, D. Vannier, and S. Marcand for critical discussion and helpful suggestions; S. Marcand for sharing unpublished results and ideas; and R. Rothstein for the use of tetrad dissection microscopes. This work was supported by grants from the National Institutes of Health (GM40094) and the American Cancer Society (VM-62) to D.S. D.W. was a recipient of a long-term fellowship from the Human Frontiers in Science Program.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

References

Aparicio, O.M., B.L. Billington, and D.E. Gottschling. 1991. Modifiers of position effect are shared between telomeric and silent mating-type loci in *S. cerevisiae*. *Cell* **66**: 1279–1287.

Blackburn, E.H. 1994. Telomeres: No end in sight. *Cell* **77**: 621–623.

Buchman, A.R., N.F. Lue, and R.D. Kornberg. 1988. Connections between transcriptional activators, silencers, and telomeres as revealed by functional analysis of a yeast DNA-binding protein. *Mol. Cell. Biol.* **8**: 5086–5099.

Buck, S.W. and D. Shore. 1995. Action of a RAP1 C-terminal silencing domain reveals an underlying competition be-

tween *HMR* and telomeres in yeast. *Genes and Dev.* **9**: 370–384.

Chi, M.-H. and D. Shore. 1995. *SUM1-1*, a dominant suppressor of *SIR* mutations in *Saccharomyces cerevisiae*, increases transcriptional silencing at telomeres and *HM* mating-type loci and decreases chromosome stability. *Mol. Cell. Biol.* **16**: 4281–4294.

Chien, C.-T., P.L. Bartel, R. Sternglanz, and S. Fields. 1991. The two-hybrid system: A method to identify and clone genes for proteins that interact with a protein of interest. *Proc. Natl. Acad. Sci.* **88**: 9578–9582.

Cockell, M., F. Palladino, T. Laroche, G. Kyrion, C. Liu, A.J. Lustig, and S.M. Gasser. 1995. The carboxy termini of Sir4 and Rap1 affect Sir3 localization: Evidence for a multicomponent complex required for yeast telomeric silencing. *J. Cell Biol.* **129**: 909–924.

Cohn, M. and E.H. Blackburn. 1995. Telomerase in yeast. *Science* **269**: 396–400.

Conrad, M.N., J.H. Wright, A.J. Wolf, and V.A. Zakian. 1990. RAP1 protein interacts with yeast telomeres in vivo: Overproduction alters telomere structure and decreases chromosome stability. *Cell* **63**: 739–750.

Drazinic, C.M., J.B. Smerage, M.C. Lopez, and H.V. Baker. 1996. Activation mechanism of the multifunctional transcription factor Repressor-Activator Protein 1 (Rap1p). *Mol. Cell. Biol.* **16**: 3187–3196.

Garvik, B., M. Carlson, and L. Hartwell. 1995. Single-stranded DNA arising at telomeres in *cdc13* mutants may constitute a specific signal for the *RAD9* checkpoint. *Mol. Cell. Biol.* **15**: 6128–6138.

Gilson, E., M. Roberge, R. Giraldo, D. Rhodes, and S.M. Gasser. 1993. Distortion of the DNA double helix by RAP1 at silencers and multiple telomeric binding sites. *J. Mol. Biol.* **231**: 293–310.

Gotta, M., T. Laroche, A. Formenton, L. Maillat, H. Scherthan, and S.M. Gasser. 1996. The clustering of telomeres and colocalization with Rap1, Sir3, and Sir4 proteins in wild-type *Saccharomyces cerevisiae*. *J. Cell Biol.* **134**: 1349–1363.

Grandin, N., S.I. Reed, and M. Charbonneau. 1997. Stn1, a new *Saccharomyces cerevisiae* protein, is implicated in telomere size regulation in association with Cdc13. *Genes & Dev.* **11**: 512–527.

Greider, C.W. 1996. Telomere length regulation. *Annu. Rev. Biochem.* **65**: 337–365.

Hardy, C.F.J. 1991. "Studies on RAP1 and the RAP1 interacting factor, RIF1." Ph.D. thesis, Columbia University, New York, NY.

Hardy, C.F.J., D. Balderes, and D. Shore. 1992a. Dissection of a carboxy-terminal region of the yeast regulatory protein RAP1 with effects on both transcriptional activation and silencing. *Mol. Cell. Biol.* **12**: 1209–1217.

Hardy, C.F.J., L. Sussel, and D. Shore. 1992b. A RAP1-interacting protein involved in silencing and telomere length regulation. *Genes & Dev.* **6**: 801–814.

Hecht, A., T. Laroche, S. Strahl-Bolsinger, S.M. Gasser, and M. Grunstein. 1995. Histone H3 and H4 N-termini interact with SIR3 and SIR4 proteins: A molecular model for the formation of heterochromatin in yeast. *Cell* **80**: 583–592.

Hecht, A., S. Strahl-Bolsinger, and M. Grunstein. 1996. Spreading of transcriptional repressor SIR3 from telomeric heterochromatin. *Nature* **383**: 92–96.

Ivy, J.M., A.J.S. Klar, and J.B. Hicks. 1986. Cloning and characterization of four *SIR* genes from *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **6**: 688–702.

Klein, F., T. Laroche, M.E. Cardenas, J.F.-X. Hofmann, D. Schweizer, and S.M. Gasser. 1992. Localization of RAP1 and

- topoisomerase II in nuclei and meiotic chromosomes of yeast. *J. Cell Biol.* **117**: 935–948.
- Krauskopf, A. and E.H. Blackburn. 1996. Control of telomere growth by interactions of RAP1 with the most distal telomeric repeats. *Nature* **383**: 354–357.
- Kurtz, S. and D. Shore. 1991. The RAP1 protein activates and silences transcription of mating-type genes in yeast. *Genes & Dev.* **5**: 616–628.
- Kyrion, G., K.A. Boakye, and A.J. Lustig. 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.
- Kyrion, G., K. Liu, C. Liu, and A.J. Lustig. 1993. RAP1 and telomere structure regulate telomere position effects in *Saccharomyces cerevisiae*. *Genes & Dev.* **7**: 1146–1159.
- Li, B. and A.J. Lustig. 1996. A novel mechanism for telomere size control in *Saccharomyces cerevisiae*. *Genes & Dev.* **10**: 1310–1326.
- Lin, J.J. and V.A. Zakian. 1995. An in vitro assay for *Saccharomyces* telomerase requires EST1. *Cell* **81**: 1127–1135.
- . 1996. The *Saccharomyces* CDC13 protein is a single-strand TG1-3 telomeric DNA-binding protein in vitro that affects telomere behavior in vivo. *Proc. Natl. Acad. Sci.* **93**: 13760–13765.
- Liu, C., X. Mao, and A.J. Lustig. 1994. Mutational analysis defines a C-terminal tail domain of RAP1 essential for telomeric silencing in *Saccharomyces cerevisiae*. *Genetics* **138**: 1025–1040.
- Longtine, M.S., N.M. Wilson, M.E. Petracek, and J. Berman. 1989. A yeast telomere binding activity binds to two related telomere sequence motifs and is indistinguishable from RAP1. *Curr. Genet.* **16**: 225–240.
- Lundblad, V. and J.W. Szostak. 1989. A mutant with a defect in telomere elongation leads to senescence in yeast. *Cell* **57**: 633–643.
- Lustig, A.J. and T.D. Petes. 1986. Identification of yeast mutants with altered telomere structure. *Proc. Natl. Acad. Sci.* **83**: 1398–1402.
- Lustig, A.J., S. Kurtz, and D. Shore. 1990. Involvement of the silencer and UAS binding protein RAP1 in regulation of telomere length. *Science* **250**: 549–553.
- Marcand, S., E. Gilson, and D. Shore. 1997. A protein-counting mechanism for telomere length regulation in yeast. *Science* **275**: 986–990.
- McEachern, M.J. and E.H. Blackburn. 1995. Runaway telomere elongation caused by telomerase RNA gene mutations. *Nature* **376**: 403–409.
- Moretti, P., K. Freeman, L. Coodley, and D. Shore. 1994. Evidence that a complex of SIR proteins interacts with the silencer and telomere-binding protein RAP1. *Genes & Dev.* **8**: 2257–2269.
- Nugent, C.I., T.R. Hughes, N.F. Lue, and V. Lundblad. 1996. Cdc13p: A single-strand telomeric DNA-binding protein with a dual role in yeast telomere maintenance. *Science* **274**: 249–252.
- Palladino, F., T. Laroche, E. Gilson, A. Axelrod, L. Pillus, and S.M. Gasser. 1993. SIR3 and SIR4 proteins are required for the positioning and integrity of yeast telomeres. *Cell* **75**: 543–555.
- Rine, J. and I. Herskowitz. 1987. Four genes responsible for a position effect on expression from *HML* and *HMR* in *Saccharomyces cerevisiae*. *Genetics* **116**: 9–22.
- Rose, M.D., F. Winston, and P. Hieter. 1990. *Methods in yeast genetics: A laboratory course manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Runge, K.W. and V.A. Zakian. 1989. Introduction of extra telomeric DNA sequences into *Saccharomyces cerevisiae* results in telomere elongation. *Mol. Cell Biol.* **9**: 1488–1497.
- Sandell, L.L. and V.A. Zakian. 1993. Loss of a yeast telomere: Arrest, recovery and chromosome loss. *Cell* **75**: 729–739.
- Schulz, V.P. and V.A. Zakian. 1994. The *Saccharomyces PIF1* DNA helicase inhibits telomere elongation and de novo telomere formation. *Cell* **76**: 145–155.
- Shampay, J. and E.H. Blackburn. 1988. Generation of telomere-length heterogeneity in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci.* **85**: 534–538.
- Shore, D. 1994. RAP1: A protean regulator in yeast. *Trends Genet.* **10**: 408–412.
- . 1995. Telomere position effect and transcriptional silencing in the yeast *Saccharomyces cerevisiae*. In *Telomeres* (ed. E.H. Blackburn and C.W. Greider), pp. 139–191. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Shore, D. and K. Nasmyth. 1987. Purification and cloning of a DNA binding protein from yeast that binds to both silencer and activator elements. *Cell* **51**: 721–732.
- Sikorski, R. and P. Hieter. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* **122**: 19–27.
- Singer, M.S. and D.E. Gottschling. 1994. *TLC1*: Template RNA component of *Saccharomyces cerevisiae* telomerase. *Science* **266**: 404–409.
- Sussel, L. and D. Shore. 1991. Separation of transcriptional activation and silencing functions of the *RAP1*-encoded repressor/activator protein 1: Isolation of viable mutants affecting both silencing and telomere length. *Proc. Natl. Acad. Sci.* **88**: 7749–7753.
- Thomas, B.J. and R. Rothstein. 1989. Elevated recombination rates in transcriptionally active DNA. *Cell* **56**: 619–630.
- Tornow, J., X. Zeng, W. Gao, and G.M. Santangelo. 1993. GCR1, a transcriptional activator in *Saccharomyces cerevisiae*, complexes with RAP1 and can function without its DNA binding domain. *EMBO J.* **12**: 2431–2437.
- Virta-Pearlman, V., D.K. Morris, and V. Lundblad. 1996. Est1 has the properties of a single-stranded telomere end-binding protein. *Genes & Dev.* **10**: 3094–3104.
- Wiley, E.A. and V.A. Zakian. 1995. Extra telomeres, but not internal tracts of telomeric DNA, reduce transcriptional repression at *Saccharomyces* telomeres. *Genetics* **139**: 67–79.
- Wright, J.H., D.E. Gottschling, and V.A. Zakian. 1992. *Saccharomyces* telomeres assume a non-nucleosomal chromatin structure. *Genes & Dev.* **6**: 197–210.
- Zakian, V.A. 1995a. *Saccharomyces* telomeres: Function, structure, and replication. In *Telomeres* (ed. E.H. Blackburn and C.W. Greider), pp. 107–137. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- . 1995b. Telomeres: Beginning to understand the end. *Science* **270**: 1601–1607.

A novel Rap1p-interacting factor, Rif2p, cooperates with Rif1p to regulate telomere length in *Saccharomyces cerevisiae*.

D Wotton and D Shore

Genes Dev. 1997, 11:

Access the most recent version at doi:[10.1101/gad.11.6.748](https://doi.org/10.1101/gad.11.6.748)

References

This article cites 54 articles, 34 of which can be accessed free at:
<http://genesdev.cshlp.org/content/11/6/748.full.html#ref-list-1>

License

Email Alerting Service

Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or [click here](#).