Rap1 in *Candida albicans*: an Unusual Structural Organization and a Critical Function in Suppressing Telomere Recombination[▽]

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Rap1 (repressor activator protein 1) is a conserved multifunctional protein initially identified as a transcriptional regulator of ribosomal protein genes in *Saccharomyces cerevisiae* but subsequently shown to play diverse functions at multiple chromosomal loci, including telomeres. The function of Rap1 appears to be evolutionarily plastic, especially in the budding yeast lineages. We report here our biochemical and molecular genetic characterizations of *Candida albicans* Rap1, which exhibits an unusual, miniaturized domain organization in comparison to the *S. cerevisiae* homologue. We show that in contrast to *S. cerevisiae*, *C. albicans* RAP1 is not essential for cell viability but is critical for maintaining normal telomere length and structure. The *rap1* null mutant exhibits drastic telomere-length dysregulation and accumulates high levels of telomere circles, which can be largely attributed to aberrant recombination activities at telomeres. Analysis of combination mutants indicates that Rap1 and other telomere proteins mediate overlapping but nonredundant roles in telomere protection. Consistent with the telomere phenotypes of the mutant, *C. albicans* Rap1 is localized to telomeres *in vivo* and recognizes the unusual telomere repeat unit with high affinity and sequence specificity *in vitro*. The DNA-binding Myb domain of *C. albicans* Rap1 is sufficient to suppress most of the telomere aberrations observed in the null mutant. Notably, we were unable to detect specific binding of *C. albicans* Rap1 to gene promoters *in vivo* or *in vitro*, suggesting that its functions are more circumscribed in this organism. Our findings provide insights on the evolution and mechanistic plasticity of a widely conserved and functionally critical telomere component.

Multifunctional Rap1 (repressor activator protein 1) was first discovered in the budding yeast *Saccharomyces cerevisiae* as a positive transcriptional regulator of multiple growth-related genes such as the ribosomal protein genes (23). Other studies identified Rap1 as the major double-strand telomere repeat binding protein in *S. cerevisiae* and to be essential for maintaining telomere length and structural integrity (5, 10). Upon further analysis, Rap1 was recognized as a key component of the mating-type silencer and shown to be required for transcriptional silencing (7, 43, 44). These disparate observations raised fascinating questions concerning the mechanisms whereby a single protein participates in diverse functions at distinct chromosomal locations. The identification of Rap1 homologues in humans and the fission yeast resulted in yet more surprises (25, 29). Both homologues were shown to be telomere-associated proteins required for proper telomere length regulation. However, instead of binding DNA directly, human and *Schizosaccharomyces pombe* Rap1 proteins were recruited to telomeres through interaction with other telomere proteins such as TRF2 and Taz1. Moreover, there is no evidence that the human and *S. pombe* proteins are involved in transcriptional regulation. Thus, although the role of Rap1 in

one particular cellular pathway appears to be conserved, its detailed molecular interactions are not.

Perhaps not surprisingly, the *S. cerevisiae* Rap1 has a complex domain architecture that befits its functional versatility. Near its N terminus is a BRCT domain, a presumed protein interaction domain whose target has not been identified. Located centrally is the DNA-binding domain, which uses a pair of Myb motifs to interact with DNA (18, 53). Interestingly, both fission yeast and human Rap1 contain just a single Myb motif, possibly accounting for their inability to bind DNA directly (25, 29). At the C-terminal end of Rap1 is a purely alpha-helical structure (RCT) that has been shown to mediate interactions with at least four other proteins required for proper telomere structure and function: Sir3, Sir4, Rif1, and Rif2 (12). Finally, a region between the DNA-binding domain and RCT has been ascribed a transcriptional activation function, although deletion of this region has little effect on the expression of some Rap1 targets (43).

Telomeres are specialized nucleoprotein structures that maintain the integrity of eukaryotic chromosomal termini by protecting them from fusion and recombination, and promoting their replication (for reviews, see references 13, 24, and 40). In most organisms, telomeric DNA consists of short repetitive sequences that are rich in G residues on the 3' end-containing strand. These repeats are maintained by a ribonucleoprotein (RNP) known as telomerase, which acts as an unusual reverse transcriptase (for reviews, see references 3, 9, and 39). Both telomere binding proteins and telomerase are critical for the maintenance of telomere integrity through multiple cell divisions, which in turn is pivotal in supporting ge-

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nome stability and promoting cellular life span. Interestingly, while the telomeres in many yeast (e.g., *Zygomycota*, *Basidiomycota*, and *Eurotiomycetes*) and metazoan species conform to the canonical TTAGGG repeat unit, the telomeres of the *Saccharomycotina* subphylum of budding yeast (including *Saccharomyces*, *Kluyveromyces*, *Dabromyces*, and *Candida* spp.) have been found to exhibit extraordinary sequence diversity (49). The length of the repeat unit ranges from 8 to 25 bp, and the repeat sequence can be rather degenerate. However, a plausible Rap1 homologue can be discerned in most of the genome databases (see below). How this protein can accommodate the variable telomere repeats in budding yeast is just one of the many unanswered questions provoked by the observation of telomere sequence divergence.

Our laboratory has utilized *C. albicans* as a model organism for understanding telomere structure and regulation (22, 46, 57). Unlike *S. cerevisiae*, this pathogenic fungus possesses a regular 23-bp telomere repeat unit. Internal to the terminal repeats are several shared subtelomeric elements, including *Care-2* and the *TLO* genes, whose functional significance has not been elucidated (51). Remarkably, in contrast to most other Rap1 homologues, the *C. albicans* gene is considerably smaller and lacks the C-terminal domain (RCT) implicated in telomere regulation (6, 50). Also in contrast to *S. cerevisiae*, two previous reports have suggested that the *C. albicans* *RAP1* is not essential for cell viability but may be involved in the regulation of pseudohyphal growth (6, 50). These studies did not address the potential role of *C. albicans* Rap1 in telomere regulation. We show here that, while not essential, *C. albicans* *RAP1* is critical for maintaining normal telomere length and structure. Consistent with a direct regulatory role, the *C. albicans* Rap1 binds with high affinity and sequence specificity to the cognate telomere repeat *in vitro* and localizes to telomeres *in vivo*. Analysis of combination mutants suggests that Rap1 plays overlapping but nonredundant roles in protecting telomeres against aberrant recombination. Furthermore, we were unable to detect specific binding of *C. albicans* Rap1 to ribosomal protein genes *in vitro* or *in vivo*, suggesting that the protein is mostly dedicated to telomere regulation in this organism. We propose a model for Rap1 evolution to account for its diverse structure and function in different budding yeast.

MATERIALS AND METHODS

Construction and growth of *Candida* strains. The *C. albicans* strain BWP17 (*ura3Δ::nim43Δ::ura3Δ::nim43Δ his1::hisG/his1::hisG arg4::hisG/larg4::hisG*) was used as the parental strain (55). Deletion *rap1*^{-/-} strains were generated by subcloning BWP17 to two rounds of transformation and 5-fluoroorotic acid selection using a *rap1::hisG-URA3-hisG* cassette (containing ~830 bp of *RAP1* upstream and ~620 bp of downstream sequence) or by using a *rap1::URA3-ARG1-URA3 (UAU1)* cassette (containing the same *RAP1* upstream and downstream sequences) (11, 16). The *rap1 sn1*, *rap1 ten1*, *rap1 ku70*, and *rap1 tert* combination deletion strains were derived from the respective single mutants by the use of the *rap1::hisG-URA3-hisG (URA-Blaster)* cassette or the *rap1::URA3-ARG1-URA3 (UAU1)* cassette. The *ku70* single mutant was kindly provided by Lidia Chico and German Larriba (Universidad de Extremadura, Spain). For the reconstituted strains containing full-length or truncated *RAP1* gene (with or without an N-terminal GSCP tag), the *rap1*^{-/-} strain was transformed with pGEM-URA3-RAP1 or pGEM-URA3-GSCP-RAP1 linearized by cleavage with HindIII. The Tbf1-TAP and Cbf1-TAP-containing strains are gifts of the Whiteway lab (27). Strains are all passaged at 30°C either in solid or liquid YPD+uri medium (2% peptone, 1% yeast extract, 2% dextrose, and 80 μg of uridine/ml). Each streak corresponds to ~2 days and ~25 generations of growth.

Plasmids and mutagenesis. A PCR fragment encompassing the entire *RAP1* open reading frame was cloned in between the NotI and SacI sites of pGEM-

URA3 to yield pGEM-URA3-RAP1. Site-specific mutagenesis was then used to create consecutive EcoRV and XhoI sites at the N terminus of *RAP1*. A triple affinity tag consisting of SBP, CBP, and protein A, followed by a Gly₆ linker, was obtained by PCR (from pGEM-URA3-TEN1-GSCP using appropriate primers) and inserted into the EcoRV and XhoI site of pGEM-URA3-RAP1 to yield pGEM-URA3-GSCP-RAP1. Finally, truncated *RAP1* genes were generated by PCR using primers containing XhoI and SacI site and used to substitute the corresponding fragment in pGEM-URA3-GSCP-RAP1.

Sequence analysis. Rap1 homologues from *Candida* and *Saccharomyces* spp. were identified from the NCBI (<http://blast.ncbi.nlm.nih.gov/blast.cgi>), Broad Institute (http://www.broad.mit.edu/annotation/genome/candida_group/broad.html), and Sanger Center (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/c_dubliniensis) databases by BLAST or psi-BLAST searches. The multiple sequence alignment was generated by using the T-COFFEE server (<http://www.igs.cnrs-mrs.fr/Tcoffee/tcoffee.cgi/index.cgi>) and displayed by using Boxshade (http://www.ch.embnet.org/software/BOX_form.html).

Telomere length analysis. Chromosomal DNAs were isolated from 3 to 5 ml of saturated *C. albicans* culture by the smash-and-grab method (19), digested with AluI and NlaIII, and fractionated in 0.6 to 0.8% agarose gels. For pulsed-field gel electrophoresis, the DNA fragments were resolved in a 1% agarose gel in 0.5× Tris-borate-EDTA at 250 V for 8 h using an initial switch time of 0.2 s and a final switch time of 0.9 s (CHEF DR II apparatus; Bio-Rad, Inc.). After transfer to nylon membranes, the telomere restriction fragments were detected as previously described by using an oligonucleotide probe that contains two copies of the *Candida* telomere repeat (45). The hybridization was performed at 50°C to 60°C. Signals from this and other studies involving radioactivity were all analyzed by using a PhosphorImager scanner and ImageQuant software (GE Healthcare, Inc.).

Two-dimensional gel electrophoretic analysis of linear and circular telomeric DNA. The two-dimensional gel analysis was performed according to the protocol of Brewer and Fangman, as modified by Cohen and Lavi (8). Briefly, the first dimension (0.5% agarose) was run at 0.5 V/cm for 16 h in the absence of ethidium bromide (EtBr). The gel was stained with 0.3 μg of EtBr/ml to visualize the size standards and bulk chromosomal DNA. Gel strips containing DNA in the 0.5- to 15-kb size range are excised and impregnated in a 1.2% agarose gel containing 0.3 μg of EtBr/ml. Electrophoresis was then performed in the orthogonal direction at 5 V/cm for 5 h. The DNAs in the gels were transferred to nylon membrane and probed with labeled oligonucleotides as described before (46).

Gel electrophoretic mobility shift analysis. *C. albicans* whole-cell extracts were prepared as previously described from 1 liter of YPD+uri culture (45). Binding reactions for unfractionated extracts contained 2 nM probe, a 1 μM concentration of nonspecific competitor oligonucleotide (5'-ACTTCTTGGTGTGGGA TGTCTA-3'/5'-TAGACATCCCAACACCAAGAAGT-3'), 2 μg of salmon sperm DNA, 1 μg of bovine serum albumin, and ~35 μg of extract in 18 μl of 10% glycerol-25 mM HEPES (pH 7.5)-5 mM MgCl₂-0.1 mM EDTA-50 mM KCl (7). For recombinant Rap1, full-length *RAP1-FLAG* was cloned into the pSMT3 vector to enable the expression of a His₆-SUMO-Rap1-Flag fusion protein. The CTG triplets encoding amino acids 76, 201, and 251 of the Rap1 protein were mutated to TCG, allowing wild-type protein to be expressed (42). After induction, extracts were prepared, and the fusion protein was purified by Ni-NTA chromatography. The fusion protein was cleaved by ULP1 protease and the Rap1-Flag fragment purified away from the His₆-SUMO tag by a second round of Ni-NTA affinity chromatography. Binding reactions for purified Rap1-Flag contained 30 nM telomeric probe in 50 mM Tris-HCl (pH 8.0), 1 mM MgCl₂, 1 mM spermidine, 1 mM dithiothreitol, and 10% glycerol. After incubation at 25°C for 20 min, the reaction mixtures were electrophoresed through a 6 to 8% nondenaturing polyacrylamide gel to resolve the free probe from the DNA-protein complex. To assess the binding of Rap1 to promoter regions, an upstream fragment of *RPL11* was amplified by PCR using two primers (5'-TG TAGATGGATAAAGTAAGTTGATT-3' and 5'-CCTTTCAAACTTTTCAA ACGAAAG-3') and used as the probe. For comparison, a 108-bp fragment of *RPL11* promoter region, in which the TBF1 and CBF1 binding sites were replaced by one telomere repeat, was used in binding reactions. This "hybrid" TELO DNA was prepared by annealing two complementary oligonucleotides (5'-aactacagtaactatgtacacaattgaaagtctctgtgtaCTTCTTGGTGTACGGATGTCTAatgttgatttttttctctgcacacattgagtttttttctta-3' and 5'-tagaaaaaaactcaatgtgtgc aggaataataatacaacatTAGACATCCGTACACCAAGAAGTAcacagatactttcaatt gttgcataatgattgtactgtagt-3'; telomere repeats are in uppercase).

Chromatin IP. Chromatin immunoprecipitation (IP) was performed using a combination of previously described protocols with some additional modifications (30, 56). Cells were fixed with 1% formaldehyde for 30 min at 30°C, and cross-linking was quenched with 125 mM glycine for 5 min at 30°C. Formaldehyde-fixed or untreated cells were resuspended in lysis buffer (50 mM HEPES

[pH 7.5], 1 mM EDTA, 150 mM NaCl, and protease inhibitors) and broken by glass beads. The lysates were sonicated 10 times for 5 s each (constant duty cycle, 35 to 40% output) to shear DNAs to a mean length of ~600 bp. Extracts were adjusted to 1.6 mg of protein/ml in 600 μ l of lysis buffer and then diluted with 600 μ l of IP dilution buffer (0.01% sodium dodecyl sulfate [SDS], 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl [pH 8.0], 450 mM NaCl, and protease inhibitors). A total of 5% of each cell extract was set aside and used as the input sample. The remainder was subjected to IP using 20 μ l of IgG-Sepharose beads at 4°C for 2 h. IP samples were washed for 5 min with rotation in the following buffers: once with buffer A (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 8.0], and 400 mM NaCl), four times with buffer B (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 8.0], and 600 mM NaCl), once with buffer C (0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, and 10 mM Tris-HCl [pH 8.0]), and once with Tris-EDTA. All of the wash buffers contained protease inhibitors. IP samples were eluted in 500 μ l of 1% SDS–0.1 M NaHCO₃, and cross-links were reversed at 65°C for 5 h. Samples were treated with RNase A and proteinase K, extracted with phenol-chloroform, precipitated with ethanol, and resuspended in 100 μ l of water. The DNA samples were then applied to Hybond-N using a dot blot apparatus, and the membrane was probed with ³²P-labeled CaC2 (5'-CATCCGTACACCAAGAAGTTAGAC ATCCGTACACCAAGAAGTTAGA-3') corresponding to two copies of the *C. albicans* telomeric repeat. *RPL11* or *RPS25* promoter fragments were also used as probes. The fragments were amplified by PCR using appropriate primers (5'-TG TAGATGGATAAAGTAAGTTGATT-3' and 5'-CCTTTCAAAACTTT TCAAACGAAAG-3' for *RPL11* and 5'-GCTCATCTGCAACTCTTTTGCC AT-3' and 5'-GTATCATTATTGAAATTAACAAAAC-3' for *RPS25*). Hybridization was performed at 68°C to minimize background.

RESULTS

Unusual structure of Rap1 homologues in *C. albicans* and related species. Two previous reports have noted the unusually small size of *C. albicans* Rap1 and its apparent loss of the well-conserved C-terminal domain (RCT) (6, 50). To determine how widespread this loss may be in evolution, we queried the NCBI and Broad Institute databases for all potential *Saccharomyces* and *Candida* homologues. Multiple sequence alignment suggests that besides *C. albicans*, the RCT of *RAP1* is completely absent from *Candida dubliniensis*, *Candida tropicalis*, and *Lodderomyces elongisporus* (Fig. 1). Moreover, this region of *C. parapsilosis* Rap1 appears to have undergone a substantial expansion. Because RCT is also present in humans and *S. pombe* Rap1, this domain is probably part of the ancient budding yeast Rap1. We therefore propose that certain *Candida* species have experienced loss or remodeling of RCT, following the branching off these species from *Candida lusitanae* and *Candida guilliermondii* (15). In contrast to the RCT divergence, each Rap1 in the *Saccharomycotina* subphylum possesses an apparent BRCT domain, as well as a duplicated Myb domain, suggesting that the interactions mediated by these domains are better conserved (Fig. 1B and data not shown).

Role of *C. albicans* Rap1 in growth and telomere regulation. To determine the function of *C. albicans* *RAP1*, we attempted to generate *rap1* null strains by using either a *UAU1*- or a *URA-Blaster*-based disruption cassette (11, 16). The former cassette can yield a null strain through one transformation step, followed by two rounds of selection, while the latter cassette requires two sequential transformation and selection steps. Consistent with previous reports, we found that *C. albicans* *rap1* null strains can be readily generated by either disruption cassette. Thus, in contrast to *S. cerevisiae*, the *C. albicans* *RAP1* gene is not essential for cell viability. The null mutant strains, however, exhibited slower growth in both solid and liquid media, forming smaller colonies and having longer

doubling times than the isogenic parental or the heterozygous strains (Fig. 2 and data not shown). Furthermore, the percentage of filamentous-form cells in rich medium is significantly higher for the null mutant (~5.5%) than for the parental strain (~0.5%). Although the reasons for this aberrant growth morphology, which was noted previously (6, 50), are not understood, similar aberrations have been described for other *C. albicans* DNA repair mutants (2, 28), suggesting a shared underlying mechanism.

Southern analysis revealed multiple interesting features of telomeric DNA in the *rap1* null mutant. First, we observed extremely long and heterogeneous telomeres in multiple isolates of the null mutant (Fig. 3A, B, and C). Long and heterogeneous telomeres were detected at the earliest time point after the derivation of the mutants (~100 generations) and were stably maintained for at least 150 generations thereafter (data not shown). In contrast to the parental BWP17, whose telomeres range in size from ca. 1 to 5 kb, the *rap1* mutants possess extremely long (>20 kb) and short (<1 kb) telomeres, a finding consistent with the loss of the homeostatic mechanism that normally regulates telomere length. Second, significant fractions of telomere signals in the mutant samples were trapped in the wells, suggesting the existence of unusual DNA structures (Fig. 3B, cf. lanes 1 and 2 with lanes 3 and 4). The reason for this is not understood, but similar structures appear to be present in the *S. pombe* *rap1* mutant (36). Finally, two-dimensional gel electrophoretic analysis revealed high levels of extrachromosomal telomeric circles (t-circles) in the null mutant (Fig. 3D). The elevated levels of t-circles in the mutant suggest that the low-molecular-weight telomeric DNA in the one-dimensional gel may be due to small circles rather than short telomeres. To test this notion, we compared the Southern hybridization patterns of untreated and restriction enzyme-digested DNA (Fig. 3B and C). The signal for low-molecular-weight telomeric DNA in the *rap1* null mutant was stronger than the wild-type strain even for untreated samples, supporting the existence of small t-circles (Fig. 3B, cf. lane 1 and 3; Fig. 3C, cf. the light and dark green trace). Then again, the low-molecular-weight signal in the mutant was further enhanced upon restriction enzyme digestion, consistent with the presence of short telomeres (Fig. 3B, cf. lane 3 and 4; Fig. 3C, cf. the light green and purple trace). We conclude that the *rap1* mutant contains an abundance of both small t-circles and short telomeres. The slow-growth, telomere heterogeneity, and t-circle accumulation phenotypes of the null strain were clearly all due to the disruption of *RAP1*; each abnormality was suppressed by the reintegration of a wild-type *RAP1* allele (Fig. 2 and Fig. 3A and D). Interestingly, both the "reconstituted" strain (which contains one copy of *RAP1*) and the heterozygous mutant possessed slightly longer telomeres than the parental strain (Fig. 3A, cf. lanes 1, 2, and 4). The *C. albicans* *RAP1* is thus likely to be haploinsufficient with respect to telomere length regulation.

Role of specific domains of *C. albicans* Rap1 in maintaining normal telomere lengths and structure. To characterize the contribution of individual domains of Rap1 to telomere regulation, we reintegrated full-length and three N-terminal truncation mutants of *RAP1* back into the null strain and examined the lengths and structure of telomeres in the resulting strains. The three truncation mutants (Δ 91N, Δ 181N, and Δ 234N)

FIG. 2. Growth of wild-type, *rap1*, and other mutant strains on plates. The indicated strains were streaked on YPD+uri medium, grown at 30°C for 2 days, and the plates were photographed.

were designed to remove successively the very N terminus, the BRCT domain, and the linker preceding the Myb domain (Fig. 4A). To facilitate biochemical analysis, the reintegrated alleles were each fused at its N terminus to a GSCP tag consisting of multiple-affinity epitopes (see Materials and Methods). Tagging of the full-length *RAP1* gene appeared to have minimal effects on its function, as judged by the similar ability of the untagged and tagged gene to suppress the growth and telomere defects of the null strain (Fig. 2 and 3A). As shown in Fig. 4B, the $\Delta 91N$ mutant Rap1 was expressed at reduced levels in multiple reintegrant strains, whereas the $\Delta 181N$ and $\Delta 234N$ were expressed at slightly higher levels, indicating that the N-terminal domains are not essential for protein stability. Interestingly, telomere Southern and two-dimensional gel analysis indicate that all deletion mutants, including the $\Delta 234N$ allele that retained just the Myb domain, were able to suppress partially the multiple telomere aberrations of the null strain. The telomeres in all of the truncation mutants were on average just 1.5 kb longer than those in the strain reconstituted with full-length *RAP1* (Fig. 4C). In addition, the levels of t-circles were reduced from ~5 to 6% of total telomeric DNA in the null strain to ~1 to 2% in the reconstituted strains (compare Fig. 3D and 4D). The deletion mutants also appeared to suppress the accumulation of complex telomere-containing structures that failed to enter the gel in Southern analysis (Fig. 4C, arrowhead). Consistent with the suppression of telomere abnormalities, all of the truncation alleles restored the growth of the *rap1* mutant to nearly that of wild-type cells (Fig. 2). Al-

together, these findings suggest that the bulk of telomeric functions of Rap1 are mediated by the putative DNA-binding Myb domain.

To determine whether the Myb domain is in fact responsible for the telomere binding activity of Rap1, we examined the ability of the truncation mutants to interact with telomeric DNA both *in vitro* and *in vivo*. First, cell extracts were prepared from the reconstituted strains and subjected to gel electrophoretic mobility shift analysis using a double-stranded telomere oligonucleotide probe (Fig. 5A). A predominant telomere DNA-specific binding activity was detected in all extracts. Consistent with being due to Rap1, the mobility of the protein-DNA complex varied as a function of the size of Rap1 protein. Notably, the levels of DNA-binding activity were similar in cell extracts derived from all of the reconstituted strains, arguing that tagging does not affect DNA binding and that the Myb domain alone is sufficient for full DNA-binding activity. Second, the ability of the Rap1 mutants to interact with telomeric DNA *in vivo* was examined by chromatin IP using IgG-Sepharose, which interacts with the protein A epitope of the GSCP tag (Fig. 5B and C). Again, the full-length and truncation mutants all exhibited tag-dependent and cross-link-dependent interaction with telomeric DNA in this assay, thus confirming their ability to localize to telomeres *in vivo*. Interestingly, the ChIP signals for the $\Delta 91N$ mutant were ~50% of those from other strains, which is consistent with the slightly reduced protein level of this mutant (Fig. 4B and 5C). We conclude that

FIG. 3. Effects of *RAP1* deletion on *C. albicans* telomere length and structure. (A) Chromosomal DNAs were isolated from the indicated strains ~75 generations after construction, digested with *AluI* and *NlaIII*, and subjected to telomere Southern analysis. (B) Chromosomal DNAs were isolated from the wild-type and a *rap1*^{-/-} strain ~75 generations after construction and subjected to telomere Southern analysis with or without prior digestion with *AluI* and *NlaIII* (+ or - RE cut). The positions of the wells are marked by an arrowhead. (C) The telomere length distributions of the indicated samples from part b were quantified by using ImageQuant and plotted. (D) Chromosomal DNAs were isolated from the indicated strains ~75 generations after construction, digested with *AluI* and *NlaIII*, and subjected to two-dimensional gel electrophoretic analysis of linear and circular telomeric DNA (indicated by arrows). The percentage of total telomeric DNA that is in the circular form is indicated at the bottom of each panel.

the Myb domain of *C. albicans* Rap1 is sufficient for telomere interaction both *in vitro* and *in vivo*.

Function of Rap1 in relation to other telomere maintenance and capping proteins. The phenotypes of the *rap1*-null mutant resemble closely those of the *ku70*, *stn1*, and *ten1* mutants; all four mutants exhibit elongated telomeres and elevated levels of t-circles (47a; L. Chico et al., unpublished data). Both *Stn1* and *Ten1* are subunits of a heterotrimeric protein complex (CST) that binds and protects telomere 3' overhangs, whereas the *Ku70*-*Ku80* complex is thought to encircle the junction between double and single stranded telomeric DNA (14, 17, 32). The phenotypic similarity between the *stn1*, *ten1*, *ku70*, and *rap1* mutants thus suggest a functional connection between proteins that interact with double-stranded and single-stranded telomeres. To further investigate the mechanisms of Rap1 in relation to other telomere protection factors, we generated *rap1 ku70*, *rap1 stn1*, and *rap1 ten1* combination mutants and compared their telomere dysfunction to those of the single mutants. As shown in Fig. 6A, all of the combination mutants exhibited long and heterogeneous telomeres resembling the single mutants. However, close analysis revealed some interesting quantitative differences (Fig. 6B and D). In the *rap1*, *stn1*, and *ten1* single mutants, the peak of telomere signals were broad and centered ~8kb, whereas in the *ku70* mutant, telomeres were somewhat less elongated. In contrast, the peaks of telomere signals in the double mutants were all larger and near

the limit of resolution for standard agarose gels (Fig. 6B and D). Pulsed-field gels confirmed that the average telomeres of the combination mutants were substantially longer than the single mutants (Fig. 6C and data not shown). Moreover, all of the combination mutants accumulated higher levels of telomere-containing DNA that were trapped in the well than did the *rap1* single mutant (Fig. 7A, arrowhead). In both the single and double mutants, the kinetics of telomere elongation was quite rapid such that the telomeres have reached their maximal lengths ~100 generations after derivation of the mutant (Fig. 7B). Similar to the telomere length aberrations, two-dimensional gel analysis indicates that the levels of t-circles in the double mutants were consistently higher than those in the corresponding single mutants (Fig. 8). The only exception was the *rap1 stn1* mutant, which did not exhibit an increase in t-circles relative to the *stn1* mutant. The possible significance of this is currently unclear. We conclude that Rap1 and other telomere protective factors mediate overlapping and nonredundant roles in regulating telomere lengths and structures.

We have shown earlier that the dramatic lengthening of telomeres in the *ten1* mutant is due to both unregulated telomerase and recombination activities (47a). To determine whether Rap1 also regulates both activities, we attempted to generate a *rap1 tert* and a *rap1 rad52* combination mutant. Interestingly, we were unable to delete *RAD52* in a *rap1* mutant using a UAU1-based cassette despite screening more than

FIG. 4. Expression levels of *RAP1* truncation mutants and their effects on telomere lengths and structure. (A) The different *RAP1* N-terminal truncation mutants analyzed in the present study are illustrated. (B) Extracts were prepared from strains containing various *RAP1* truncation alleles and subjected to Western analysis with antibodies against protein A. Three independently derived clones for each genotype were assayed. (C) Chromosomal DNAs were isolated from strains containing various *RAP1* truncation alleles and subjected to telomere Southern analysis following three streaks on solid media. The positions of the wells are marked by an arrowhead. Three independently derived clones for each genotype were assayed. (D) Chromosomal DNAs were isolated from strains containing various *RAP1* truncation alleles and subjected to two-dimensional gel analysis of telomeric DNA after three streaks on solid media. The level of t-circles as percentage of total telomeric DNA for each sample is indicated at the bottom of the panel.

30 Arg⁺ Ura⁺ colonies (11). This observation suggests that disruption of *RAD52* and *RAP1* may be synthetically lethal. On the other hand, several *rap1* *tert* combination mutants can be readily constructed and these mutants exhibited telomeres that

are slightly more elongated than the *rap1* single mutant (Fig. 6A and E). This observation implies that aberrant recombination is primarily responsible for the telomere lengthening of the *rap1* mutant. Curiously, whereas deletion of *TERT* (named

FIG. 5. DNA-binding activity of *Rap1* truncation mutants *in vitro* and their localization to telomeres *in vivo*. (A) Whole-cell extracts were prepared from the indicated *C. albicans* strains and subjected to gel electrophoretic mobility shift analysis by using a double-strand telomere probe. Unlabeled telomeric DNA was added at a 500-fold excess to some reactions as indicated to test the specificity of the complex. The positions of the free probe and DNA-protein complex are marked by an arrowhead and a bracket, respectively. (B) Strains containing various *RAP1* truncation mutants were subjected to chromatin IP analysis. The input and IP samples were analyzed using a telomere probe. (C) The levels of telomeric DNA in the IP as percentages of input telomeric DNA were calculated and plotted. The data displayed are means $\pm$ the standard deviations (SD) from three independent assays.

FIG. 6. Phenotypes of the *rap1* combination mutants. (A) Chromosomal DNAs were isolated from various mutant strains and subjected to telomere Southern analysis after approximately three streaks on solid media. Two independent clones of each double mutant were analyzed. (B) The length distributions of telomeres for the indicated mutants as revealed in the Southern analysis shown in Fig. 6A are analyzed by using ImageQuant software and plotted. (C) Telomeres in the indicated mutants strains were released by restriction enzyme digestion and analyzed by CHEF gel electrophoresis and Southern hybridization. (D and E) The length distributions of telomeres for the indicated mutants as revealed by the Southern analysis shown in panel A are analyzed by using ImageQuant software and plotted.

for telomerase reverse transcriptase) in a wild-type strain does not induce t-circle formation, loss of *TERT* in a *rap1* strain results in a further elevation in the levels of t-circles (Fig. 8). Thus, telomerase may play a minor role in suppressing telomere recombination such that the function is only revealed in the absence of other protective factors. An analogous function has been attributed to the *S. pombe* homologue Trt1 (47).

Specific recognition of the unusual *C. albicans* telomere repeat unit by *C. albicans* Rap1. As noted before, the sequences of telomere repeat units in the *Saccharomyces* and *Candida* clades are exceptionally diverse, and yet each genome appears to contain a convincing Rap1 homologue, thus raising interesting questions with regard to the mechanisms of DNA recognition by this protein. Besides *S. cerevisiae* Rap1, which has been exhaustively studied, only the binding specificity of the *Saccharomyces castellii*, *Saccharomyces dairensis*, and *Kluyveromyces lactis* homologues have been examined to some degree using partially purified preparation (26, 52–54). We thus undertook a detailed characterization of the DNA-binding properties of *C. albicans* Rap1 to gain insights into the mechanisms of telomere recognition. Full-length *C. albicans* Rap1 with a C-terminal FLAG tag was expressed as a SUMO-fusion pro-

tein in *E. coli*. After removal of the SUMO tag and further purification, the Rap1-FLAG protein was subjected to a series of EMSA analysis to determine its binding affinity and sequence specificity (Fig. 9). As expected, the *C. albicans* Rap1 protein binds to the *C. albicans* telomere repeat with high affinity ($K_{d\text{ app}} = \sim 15$ nM) (Fig. 9C). The protein has clearly evolved preference for the cognate telomere repeat unit, because the purely GT repeat of the *S. cerevisiae* telomere and the more divergent *C. pseudotropicalis* repeat do not compete efficiently for binding (Fig. 9C and D). On the other hand, the more closely related *C. tropicalis* and *C. maltosa* telomere repeat do compete efficiently, indicating that the recognition property of Rap1 is not entirely species specific (Fig. 9C and D).

To define further the sequence specificity of the *C. albicans* Rap1 protein, we used a series of mutant oligonucleotides, each bearing two or three nucleotide substitutions, as competitors in the binding assays (Fig. 9B). As shown in Fig. 9E and F, two GT-rich pentanucleotide elements within the telomere repeat unit (GGTGT and GGATG) appear to be most important for sequence-dependent recognition. Substitutions that overlap with these sequence elements all had moderate or

FIG. 7. Characteristics of telomere elongation in the *rap1* and other combination mutants. (A) Chromosomal DNAs were isolated from the indicated strains and assayed for telomere length distributions as described. The positions of the wells are indicated by an arrowhead. (B) Chromosomal DNAs were isolated from the indicated strains after three successive passages on plates and assayed for telomere length distributions as indicated. Two independently derived *rap1 stn1* and *rap1 ten1* mutants were analyzed.

severe effects on the abilities of the mutant oligonucleotides to serve as competitors. Notably, the result of this analysis is in excellent agreement with that using heterologous telomere repeats (Fig. 9C and D). For example, the differences between

FIG. 8. Levels of t-circles in various telomere mutants. The levels of t-circles as percentages of total telomeric signals are determined and plotted. The data are means $\pm$ the SD from three or four samples as indicated. Except for the minimal difference between the *rap1 stn1* combination mutant and the *stn1* mutant, each combination mutant exhibited a statistically significant increase in t-circle level over the corresponding two single mutants (Student *t* test, *P* < 0.05).

the *C. albicans*, *C. tropicalis*, and *C. maltosa* telomere repeat lie entirely outside of the two pentanucleotide elements, thus accounting for the abilities of the *C. tropicalis* and *C. maltosa* repeat to compete efficiently in the binding assays. To assess other features of the telomere sequence necessary for high-affinity interaction with Rap1, we investigated three other permutations of the telomere repeat unit (Fig. 10A). One permutation (“b”) reversed the order of the two elements and increased their spacing by 9 bp. This oligonucleotide did not interact with Rap1 with appreciable affinity (Fig. 10B). Two other permutations (“c” and “d”) retained the spatial relationship between the elements but place one of the elements at the edge of the oligonucleotide. Both of these permutations exhibit reduced binding to Rap1, with oligonucleotide “d” showing a more severe defect (Fig. 10B and C). We conclude that the spatial relationship between the pentanucleotide elements is important for Rap1 binding. In addition, even though nucleotides surrounding of the pentanucleotide elements appear not to be involved in sequence-dependent recognition, they may contribute to the interaction affinity.

Lack of interaction between *C. albicans* Rap1 and gene promoters *in vitro* and *in vivo*. Besides our observation of robust telomere binding, the *C. albicans* Rap1 has previously been reported to bind an RPG box located in the promoter of the *S. cerevisiae* *ENO1* gene, a finding consistent with a potential role in transcriptional regulation (50). However, this sequence element, which exhibits little similarity to the *C. albicans* telomere repeat unit, failed to interact with our purified *C. albicans*

FIG. 9. DNA-binding specificity of *C. albicans* Rap1 in vitro. (A) Approximately 5 μ g of purified recombinant Rap1-FLAG was analyzed by SDS-PAGE and Coomassie blue staining. (B) The sequences of the oligonucleotides used in the binding assays are displayed. The two pentanucleotide elements shown by the assays to be recognized specifically are indicated in boldface. Only the top strands are displayed. (C) Purified recombinant Rap1 was subjected to EMSA analysis using the *C. albicans* telomere repeat (30 nM) as the probe and the indicated competitors. The concentrations of Rap1 used in individual assays are shown at the top of the panel. Competitor oligonucleotides are included at 75 and 300 nM. (D) Purified recombinant Rap1 (5 nM) was subjected to electrophoretic mobility shift assay (EMSA) analysis using the *C. albicans* telomere repeat (30 nM) as the probe and various unlabeled oligonucleotides (600 nM) as competitors. (E) Purified recombinant Rap1 (20 nM) was subjected to EMSA analysis by using the *C. albicans* telomere repeat (30 nM) as the probe and the indicated mutant oligonucleotides as competitors (600 nM). (F) The relative amounts of protein-DNA complex from experiments such as those shown in E were quantified and plotted. The data are means $\pm$ the SD from three independent experiments. Statistically significant differences between the competition activity of the mutant and wild-type oligonucleotide ($P < 0.05$) are indicated by asterisks.

FIG. 10. Analysis of the DNA-binding property of *C. albicans* Rap1. (A) The sequences of the oligonucleotides used in this analysis are listed. The two pentanucleotide elements within the telomere repeat that are recognized specifically by the protein are shown in bold or underlined. (B) Purified recombinant *C. albicans* Rap1 (0, 5, 10, and 20 nM) was subjected to EMSA analysis using different permutations of the telomere repeat unit as the probe (30 nM). A low-level, contaminating complex is sometimes observed in these assays (indicated by an asterisk). (C) Purified recombinant *C. albicans* Rap1 (10 nM) was subjected to EMSA analysis using oligonucleotide "a" as the probe (30 nM) and different permutations of the telomere repeat unit as competitors (600 nM).

Rap1 (data not shown). Interestingly, a recent study by Hogue et al. proposes that the transcriptional activation function of *Saccharomyces* Rap1 at ribosome-related genes is relatively modern and is probably not conserved in *Candida* species (20). These investigators argue instead that the *Candida* ribosomal genes are activated by two other transcription factors, Tbf1 and Cbf1. To reinvestigate this issue, we analyzed the interaction between *C. albicans* Rap1 and the *RPL11* or *RPS25* promoter. Both *S. cerevisiae* *RPL11* and *RPS25* are known to be regulated by Rap1, but their homologues in *C. albicans* are proposed to be under the control of Tbf1 and Cbf1 (20). We were unable to detect a DNA-protein complex between Rap1 and the *RPL11* promoter (Fig. 11A, right panel), even though a hybrid DNA probe containing a telomere repeat unit inserted into a subfragment of the promoter yielded a clear complex (Fig. 11A, left panel). We then attempted to detect localization of Rap1 upstream of the *RPL11* and *RPS25* genes by ChIP. Despite robust signals for telomeric DNA, the levels of *RPL11* and *RPS25* promoters in the cross-linked Rap1 IP samples were extremely low, suggesting that Rap1 does not associate with these promoters *in vivo* (Fig. 11B). In contrast, we were able to demonstrate clear localization of *C. albicans* Tbf1 and Cbf1 to the *RPL11* promoter (Fig. 11C). Thus, our findings are indeed consistent with a more dedicated role for Rap1 at telomeres in *C. albicans*.

DISCUSSION

***C. albicans* Rap1 cooperates with other telomeric proteins to suppress aberrant recombination activity at telomeres.** We have shown that despite its unusual, "miniaturized" domain organization, the *C. albicans* Rap1 has important functions in suppressing aberrant telomere elongation and t-circle formation. Both phenotypes appear to be due to abnormal recombination at telomeres because the *rap1 tert* combination mutant exhibits the same features. Our findings thus demonstrate for the first time a novel function for Rap1 in suppressing telomere recombination. Notably, *K. lactis* mutants with aberrant telomere repeats that cannot be bound by Rap1 have also been reported to experience high levels of telomere recombination (4). It is thus tempting to suggest that Rap1 mediates a conserved function in regulating abnormal telomere recombination in *C. albicans* and *K. lactis*. Furthermore, analysis of other telomere protein mutants suggests that this regulatory activity of Rap1 is shared by other telomere proteins such as Ku70, Stn1 and Ten1 (47a; Chico et al., unpublished). Although there appears to be some functional overlap between these telomere protection factors, they are clearly not redundant because combination mutants almost invariably have exacerbated phenotypes relative to single mutants (Fig. 6, 7, and 8). It is also worth noting that these phenotypes are reminiscent of ALT cancer cells, which evidently maintain telomeres through re-

FIG. 11. Interaction of Rap1 with gene promoters. (A) Purified recombinant *C. albicans* Rap1 was subjected to EMSA analysis using either a “hybrid” telomere DNA (TELO) or an RPL11 promoter fragment (RPL11) as the probe. The preparations of the DNAs are described in Materials and Methods. The positions of the free probes and the DNA-protein complex are marked by arrowheads and a circle, respectively. (B) *C. albicans* strains containing untagged or GSCP-tagged Rap1 were subjected to chromatin IP using IgG-Sepharose. The input and precipitated DNA were probed with a telomere oligonucleotide (top) and an *RPL11* or *RPL25* fragment (bottom). Because of the lower copy number of the ribosomal protein gene in the genome, a fourfold-higher input and IP samples were used for the *RPL11/RPL25* analysis. (C) *C. albicans* strains containing untagged or protein A-tagged Rap1, Tbf1, and Cbf1 were subjected to chromatin IP using IgG-Sepharose. The parental strains into which the tagged alleles were inserted are listed at the top. Because the tagged *RAP1* strain was made by inserting the tagged allele into a *rap1*-null strain, we also analyzed a strain reconstituted with untagged *RAP1* for comparison. The input and precipitated DNA were probed with an *RPL11* promoter fragment.

combination (37, 38). Altogether, our data suggest that disrupting either the double-strand or the single-strand telomere nucleoprotein complex can lead to an ALT-like state and that the *C. albicans* mutants may be useful model systems for understanding the molecular basis of ALT.

Another interesting defect of *C. albicans rap1* mutant is the accumulation of aberrant telomere-containing DNA structures that cannot enter standard agarose gels. This phenotype appears to be manifested by the *S. pombe rap1* mutant as well (36). Interestingly, we observed a slight increase in the level of single-stranded telomere DNA by in-gel hybridization, but the single-stranded DNA signals are mostly resistant to ExoI digestion (data not shown). It is tempting to speculate that this phenotype may be another manifestation of the same aberrant telomere DNA structures.

At *S. cerevisiae* telomeres, Rap1 has been demonstrated to serve at least two critical functions: suppressing telomere-telomere fusion (t-t fusion) and regulating telomerase activity through a “protein-counting” system (31, 33, 34). Our initial attempts at detecting t-t fusions in *C. albicans* wild-type and *rap1* null strains using the PCR strategies developed previously for *S. cerevisiae* were unsuccessful (data not shown) (35, 41). In addition, as mentioned before, we were unable to obtain strains missing both *RAP1* and *RAD52*. Consequently, we are at present unable to determine whether the two well-established functions of *S. cerevisiae* Rap1 are also performed by the

C. albicans homologue. Further studies will be necessary to address these issues. However, it is noteworthy that both functions of *S. cerevisiae* Rap1 are mediated in part by its RCT domain, which is missing in the *C. albicans* homologue. Thus, it would not be surprising if some functional disparity has arisen between the two Rap1 proteins.

A particularly intriguing observation that emerged from our analysis of combination mutants concerns the possible function of telomerase in regulating telomere recombination. In particular, while the *tert* single mutant exhibited telomere shortening and no detectable increase in t-circles, the *rap1 tert* combination mutant evidently manifested a more severe telomere lengthening and t-circle accumulation phenotype than the *rap1* single mutant (Fig. 6, 7, and 8). Thus, it is tempting to speculate that telomerase may play a minor and partly redundant role in suppressing telomere recombination. This possibility is consistent with the conclusion of a recent study in *S. pombe* (47). Moreover, we have previously demonstrated an enzyme activity-independent function of telomerase in telomere protection, which may be related mechanistically to its function in regulating recombination (21).

Mechanisms of DNA-binding by *C. albicans* Rap1 and implications for telomere evolution. Previous analysis of DNA-binding by the Rap1 proteins from several *Saccharomyces* species has led to the proposal that the two Myb domains recognize two G-rich sequence elements within the diverse

FIG. 12. Evolution of Rap1 in budding yeast. A possible series of evolutionary events that resulted in the current diversity in Rap1 structure and function are illustrated. In the ancestral yeast, Rap1 was proposed to be tethered to telomeres through an interaction with TRF2. During evolution, an alteration in the telomere repeat sequence led to the loss of TRF2 and other associated factors. Subsequently, Rap1 evolved new DNA-binding activities, possibly through the duplication of its Myb domain, to protect the double-stranded telomere DNA. No longer required for telomere localization, the original RCT domain of Rap1 was eventually either lost in some descendants or evolved new functions in others.

telomere repeat units of budding yeast (52–54). Variable distances between the two core elements of different binding sites are accommodated due to a flexible linker between the Myb domains. Moreover, crystal structures of Rap1 bound to different target sites demonstrate that the protein can also use subtle side chain movements at the protein-DNA interface to recognize diverse sequence (48). Thus, the challenge posed by the diverse telomere repeat units in budding yeast could be met by Rap1 through a variety of means without major changes in the DNA-binding residues. A corollary of this hypothesis is that all of the *Candida* and *Saccharomyces* Rap1 may have very similar and flexible DNA-binding properties. This notion is supported by multiple sequence alignment of the Rap1 Myb domain; amino acid residues implicated in contacting DNA bases are mostly conserved between *Saccharomyces* and *Candida* Rap1 (Fig. 1B). Also consistent with this notion is our finding that the critical sequence determinants for *C. albicans* Rap1 binding (i.e., the two pentanucleotide elements [GGTGT and GGATG]) are shared by most telomeres of the *Candida* clade. On the other hand, some species specificity has clearly emerged during evolution such that the *S. cerevisiae* and *C. pseudotropicalis* telomere repeats interact with the *C. albicans* protein with reduced affinity. In this regard, it would be especially interesting to determine whether the *C. pseudotropicalis* Rap1 has acquired amino acid changes in order to recognize preferentially the corresponding telomere repeat, which contains a variant pentanucleotide element (GGATT rather than GGATG; see Fig. 9B). Clearly, more studies will be necessary to arrive at a detailed understanding of the molecular basis of DNA-binding by *Candida* Rap1.

Rapid evolution of Rap1 in *S. cerevisiae* and *C. albicans*. The dispensability of *C. albicans* RAP1 for cell viability is in accord with previous observations and consistent with current views on the essential role of RAP1 in *S. cerevisiae*. Notably, an *S. cerevisiae* strain with “humanized” telomeres that fail to bind Rap1 remains viable, indicating that the telomere function of this gene is not essential for *S. cerevisiae* cell viability (1). Instead, the transcriptional activation function of Rap1 may account for its indispensability. Thus, the nonessential nature of *C. albicans* RAP1 can be rationalized by a more limited role in transcriptional regulation. Interestingly, a recent study suggests that much of the transcriptional activation function of Rap1 evolved recently, after the *Saccharomyces* and *Kluyveromyces* species branched off from other yeast (20). In the *Kluyveromyces* and *Saccharomyces* genera, Rap1 acquired a new function in upregulating the transcription of many growth related genes, including the ribosomal protein and RNA genes. In contrast, the equivalent function in *C. albicans* and other budding yeast is evidently mediated by two other transcription factors named Tbf1 and Cbf1. Indeed, we were unable to detect an interaction between *C. albicans* Rap1 and two ribosomal protein gene promoters (Fig. 11). Moreover, genome-wide localization analysis of *C. albicans* Rap1 revealed a much more limited presence of this protein relative to its *Saccharomyces* counterpart, which is consistent with a more dedicated role of *C. albicans* Rap1 in telomere regulation (H. Lavoie et al., personal communication). Thus, the different range of *in vivo* function for Rap1 can account for the differential effect of RAP1 deletion on cell viability.

Both the telomere and the transcription-related functions of

Rap1 have evidently experienced substantial evolutionary changes in budding yeast. It is tempting to speculate that these changes are both consequences of the unusual telomere repeat divergence in these organisms. A possible series of past events that can rationalize Rap1 evolution is presented in Fig. 12. First, we hypothesize that in the ancestral budding yeast, Rap1 was tethered to telomeres through an interaction between its RCT domain and TRF2 (as in humans) and mediated a crucial telomere protection function. In accordance with earlier proposals (29, 49), we imagine that multiple telomerase RNA template mutations were somehow fixed in descendants of the yeast. The corresponding telomere sequence alterations in turn caused a major remodeling of the telomere nucleoprotein complex. This remodeling entails both the loss of many canonical telomere binding proteins including TRF2 and the acquisition of a direct-telomere DNA-binding activity by Rap1. Possibly, an accidental duplication of the Myb domain of Rap1 conferred the protein with enhanced potential for DNA binding. Because the RCT of Rap1 was no longer needed for its telomere localization, there was less selection pressure for its retention. The RCT was also in principle free to evolve new interactions and binding partners that might confer selective advantage to the organism. Thus, both the loss of RCT and the acquisition of new activities by RCT (i.e., the Rif1, Rif2, Sir3, and Sir4-interaction activity and the transcription activity of Rap1) in different yeast lineages could be considered natural consequences of the relaxing of constraints on RCT function.

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