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RPA regulates telomerase action by providing Est1p access to chromosome ends

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Replication protein A (RPA) is a highly conserved single-stranded DNA–binding protein involved in DNA replication, recombination and repair. We show here that RPA is present at the telomeres of the budding yeast *Saccharomyces cerevisiae*, with a maximal association in S phase. A truncation of the N-terminal region of Rfa2p (associated with the *rfa2Δ40* mutated allele) results in severe telomere shortening caused by a defect in the *in vivo* regulation of telomerase activity. Cells carrying *rfa2Δ40* show impaired binding of the protein Est1p, which is required for telomerase action. In addition, normal telomere length can be restored by expressing a Cdc13-Est1p hybrid protein. These findings indicate that RPA activates telomerase by loading Est1p onto telomeres during S phase. We propose a model of *in vivo* telomerase action that involves synergistic action of RPA and Cdc13p at the G-rich 3′ overhang of telomeric DNA.

Replication protein A (RPA) is a eukaryotic single-stranded DNA–binding protein involved in processes including replication, recombination, transcription, checkpoints, telomere maintenance and DNA repair[1–3]. Human and yeast RPA are each composed of three subunits of 70, 32 and 14 kDa[4]. RPA70 and RPA32/34 (in yeast Rfa1p and Rfa2p) become phosphorylated in response to DNA damage[5,6]. After DNA damage, phosphorylation of Rfa1p depends on the checkpoint proteins Mec1p, Mec3p, Rad9p and Rad53p whereas phosphorylation of Rfa2p depends mainly on Mec1p[5,6]. Rfa2p is also phosphorylated during the normal cell cycle—in a process that depends on the yeast homologs of the related PI3-like protein kinases ataxia-telangiectasia mutated (ATM) and ATM-Rad3-related (ATR) Mec1p and Tel1p (ref. 5)—and during meiosis[7]. Finally, we have shown that inactivation of the histone H3 methyltransferase Set1p induces a *MEC1*- and *TEL1*-independent hyperphosphorylation of Rfa2p in G1 phase in a process that depends on the checkpoint kinase Rad53p (ref. 8). Notably, in *set1Δ* cells, a truncated form of Rfa2p lacking 38 amino acids from the N terminus of Rfa2p (Rfa2pΔ40), is still phosphorylated in S phase by Mec1p but is not phosphorylated in G1 phase (ref. 8). The *rfa2Δ40* mutation conveys checkpoint proficiency, increased viability of checkpoint mutants subjected to irradiation, and slightly slower DNA replication (a delay of 15 min as compared to wild type)[8].

Telomeres are essential to the stability of chromosome ends. In most organisms, including yeast and vertebrates, telomeric DNA consists of a tandem array of short guanine-rich repeats[9]. The telomeric repeats are synthesized by a unique ribonucleoprotein reverse transcriptase called telomerase, which specifically extends the 3′ G-rich telomeric strand[10]. Synthesis of the complementary 5′ C-rich telomeric strand is thought to be driven by the DNA polymerase α–primase complex[11]. In *S. cerevisiae*, the 3′ ends of telomeric DNA consist of a G-rich single-stranded stretch that is detected only during S phase[12]. This G-tail associates with two single-stranded DNA–binding proteins, Cdc13p and Est1p, that are required for telomerase activity *in vivo*[13–15]. The action of telomerase at the telomere itself is cell-cycle restricted, indicating that a switch in telomere structure may be required for telomerase action[16–19]. This process is related to the interaction of the telomerase catalytic subunit (Est2p) with Cdc13p and Est1p (refs. 14,15,18). Est2p seems to be present at telomeres throughout the cell cycle, in contrast to Est1p, which binds telomeres mainly in S phase in a fashion that seems to be preserved in the presence of the defective *cdc13-2* allele[18]. It has therefore been proposed that Est1p binds telomeres in late S phase and interacts with Cdc13p to activate telomere-bound Est2p (ref. 18).

Here we investigated the relationships between RPA and telomeres. We show that RPA is required to maintain telomere length and acts in a pathway that involves Est2p and the telomerase positive regulator Tel1p. Our results indicate that RPA regulates the action of telomerase during the cell cycle by specifically facilitating the binding of Est1p to telomeres during S phase.

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RESULTS

Binding of Rfa2p to telomeric DNA

We investigated whether Rfa2p is bound *in vivo* to telomeric DNA. We first used a one-hybrid assay for telomere-binding proteins[20] in which Rfa2p was fused to a transcriptional activation domain and tested for its ability to activate transcription of a telomere-linked, promoter-defective *HIS3* allele. We compared transcriptional activation of the telomeric *HIS3* reporter by the Rfa2p fusion protein to transcriptional activation by similar fusion proteins of Rif2p (Rif2p binds telomeres through its interaction with the telomeric DNA binding protein Rap1p) and of Cdc13N (the N-terminal domain of Cdc13p, which does not contain the DNA-binding domain). The Rfa2p fusion protein induced transcriptional activation of the telomere-linked *HIS3*, indicating that Rfa2p is a telomere-binding protein (**Fig. 1a**). The Rfa2p fusion protein produced weaker transcriptional activation than the Rif2p fusion protein but stronger activation than the Cdc13N fusion protein. Unlike Rif2p, Rfa2p did not activate the *HIS3* reporter gene when it was adjacent to an internal tract of telomeric sequences (**Fig. 1a**). This indicates that *in vivo* binding of Rfa2p may require the presence of a chromosome end.

To confirm the association of Rfa2p with telomeric DNA, we carried out chromatin immunoprecipitation (ChIP) assays. We used cells carrying the *URA3* reporter inserted next to the left telomere of chromosome VII and expressing a Myc-tagged version of Rfa2p (UCC1001 *RFA2-MYC*). We considered the Rfa2-Myc fusion protein to be functional: the *RFA2-MYC* cells behaved like wild-type cells with respect to growth, DNA replication, biological responses to DNA damage and telomeric position effects, although their telomeres were slightly (30 bp) longer than those of wild-type cells (data not shown). Chromatin from *RFA2-MYC* cells was cross-linked, immunoprecipitated and analyzed by PCR. Rfa2-Myc immunoprecipitates allowed amplification of the telomeric PCR products pUra1 and pUra2, or pTelVI, but not of the control pBdf1 PCR fragment (**Fig. 1b**). When cells without the Myc-tagged Rfa2p were subjected to the same ChIP assay, or when immunoprecipitation was done in the absence of *in vivo* formaldehyde cross-linking, we did not detect any PCR amplification (**Fig. 1b**), demonstrating the specificity of the ChIP.

Next we addressed whether Rfa2p showed a cell cycle–regulated association with telomeres. We released cells expressing the chromosomally

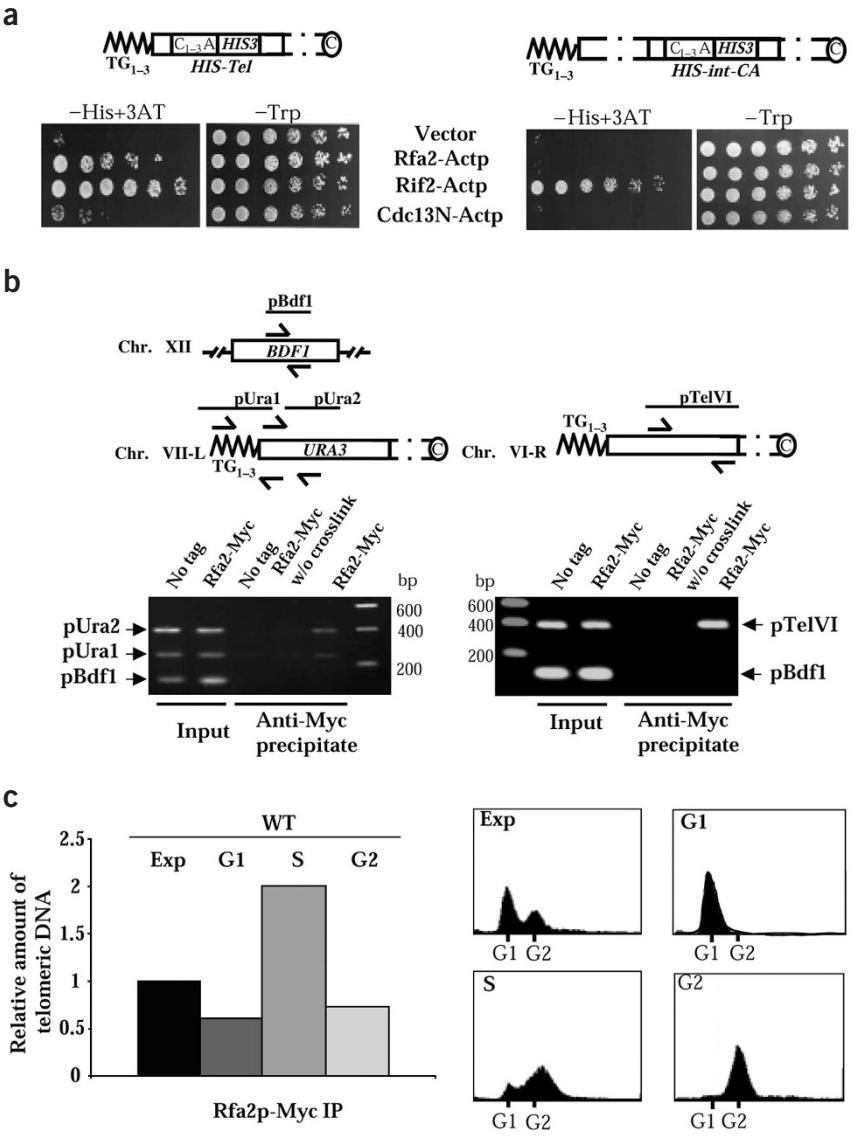

Figure 1 Rfa2p binds to telomeric DNA *in vivo*. (**a**) Rfa2p fusion protein activates HIS3 at a telomere but not at an internal tract of telomeric DNA. Proteins fused to the B42 activation domain were expressed in strains (YM701 background) in which the *HIS3* promoter–defective allele was inserted, either adjacent to an internal tract of telomeric DNA (HIS-Int-CA) or immediately adjacent to the left telomere of chromosome VII (HISTel)[20]. Cells were spotted onto test plates (–His+3AT) or control plates (–Trp). (**b**) Scheme showing the fragmented *URA3* gene at the left arm of chromosome VII (left), the telomere at the right arm of chromosome VI (ChR-VI; right) and *BDF1* at chromosome XII (top). Primer pairs used to amplify telomeric fragments pUra1 and pUra2, Chr-VI-R telomere fragment pTelVI and control nontelomeric fragment pBdf1 are indicated. The ChIP assay was carried out with antibodies to Myc (9E10) on chromatin from the indicated UCC1001 strains. After DNA purification, PCR was carried out with telomeric primers (pUra1, pUra2 and pTelVI) and nontelomeric control primers (pBdf1). (**c**) Association of Rfa2p with telomeric DNA increases in S phase. Right, FACS analysis of the synchronized UCC1001 cells after release from α-factor-induced G1 block. Left, results of ChIP assays on Myc-tagged Rfa2p, carried out on chromatin from cells harvested in exponentially growing (exp), G1 phase, S phase and G2 phase cells after release from an α-factor block. Relative enrichment of Rfa2-Myc-bound telomeric DNA (pUra2) over background (pBdf1) was calculated as indicated in Methods. The relative amount of telomeric DNA in the precipitates of exponentially growing cells was arbitrarily taken as 1.

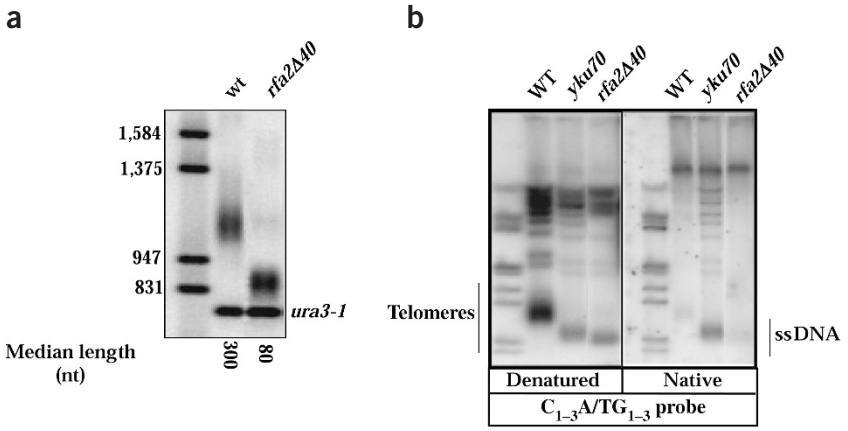

Figure 2 Rfa2p is involved in telomere length regulation. (**a**) Comparison of the telomere length of haploid strains isogenic to UC1001 grown for more than 100 generations carrying a subtelomeric *URA3* reporter gene on the left arm of chromosome VII. Median telomere length was calculated from two independent experiments using ImageQuant (Molecular Dynamics). (**b**) The *rfa2Δ40* mutation does not lead to an increase in single-stranded telomeric G-rich DNA. Genomic DNAs isolated from the indicated strains were digested with *Xho*I and analyzed by nondenaturing hybridization (native) followed by denaturation of the DNA (denatured) in the same gel and rehybridization with the same telomeric C1–3A/TG1–3 probe as described[21]. nt, nucleotide; ss, single-stranded; WT, wild type.

encoded Rfa2-Myc18 from an α-factor-induced G1 block and monitored their progression through the cell cycle by FACS analysis (**Fig. 1c**). Cells in G1, S and G2 phases were subjected to ChIP (**Fig. 1c**). Amounts of DNA in the input samples and immunoprecipitates were measured by real-time PCR and normalized to the level of the nontelomeric pBdf1 fragment presents in the immunoprecipitates. PCR quantification analysis indicated that the amount of telomeric DNA bound to Rfa2p in cells harvested in S phase was four times higher than in cells harvested in G1 phase (**Fig. 1c**). Data showing enrichment at telomeres, as compared to a nontelomeric control of Rfa1p and Rfa2p during the cell cycle, is described below.

Rfa2p is involved in regulation of telomere length

We had previously constructed a mutant strain with a chromosomally encoded, truncated form of Rfa2p lacking 38 amino acids from the N terminus[8]. We introduced the mutated allele, *rfa2Δ40*, into yeast strain UCC1001, which carries a subtelomeric *URA3* reporter gene, and examined the median length of the left telomere of the fragmented chromosome VII-L. Telomeres in the *rfa2Δ40* strain were critically short (220 bp shorter than wild-type telomeres; **Fig. 2a**). We next asked whether this telomere shortening was accompanied by an increase in the amount of single-stranded G-strands in exponentially growing cells, as is the case for cells deficient for Ku *(yku)*[21], a DNA-binding heterodimer that is crucial in DNA end protection. In wild-type cells, telomeric single-stranded extensions cannot be detected with telomeric probes in the native DNA of cells outside S phase[12]. This contrasts with *yku* mutants, which show long terminal extensions of the G-rich strand. We did not detect long G-tails in the *rfa2Δ40* cells, although we detected them in *yku70* mutants. Natural telomeres were also shortened in *rfa2Δ40* cells (**Fig. 2b**).

RPA acts in the telomerase pathway

The length of the single-stranded overhang has been proposed to correlate with the rate of telomeric DNA degradation[22]; thus, the absence of an expanded 3′ overhang in *rfa2Δ40* suggested that this allele does not affect the rate of telomeric DNA degradation. To address this prediction directly, we compared telomere length dynamics in Rfa2p and Est2p mutants. We constructed a heterozygous diploid strain (JKM179 background) carrying the *rfa2Δ40* N-terminal truncation allele and a deletion allele of *EST2*, *est2Δ*. The diploid strain *RFA2/rfa2Δ40*; *EST2/est2Δ:NAT*, *ADH4/TELadh4:URA3* was sporulated, and the growth reduction with increasing

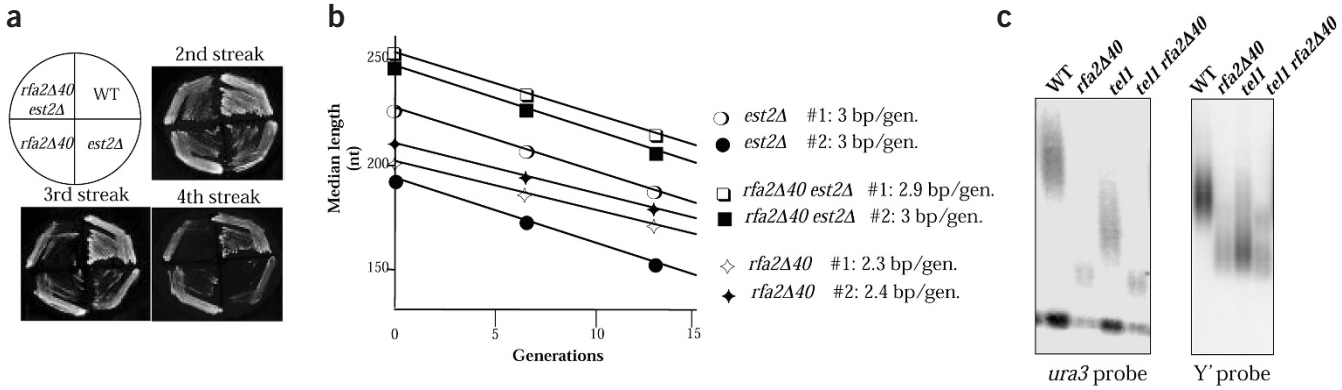

Figure 3 *RFA2* acts in the telomerase pathway. (**a**) Viability of the *est2*Δ, r*fa2Δ40* and *est2*Δ *rfa2Δ40* strains with increasing number of generations (gen.). Shown are successive streak cultures on YPD medium of spores generated from a single tetrad. (**b**) Telomere degradation rates. Telomere length of the indicated yeast strains (JKM179 background) were plotted against generation and telomeric degradation rates were calculated from linear regression curves. (**c**) Deleting *TEL1* does not aggravate telomere shortening of the *rfa2Δ40* mutant. Telomere length of the indicated spores was measured after 3 streakings. Left, the *URA3* genetically marked telomere was analyzed as in **Figure 2a**. Right, lengths of Y′ telomeres. Telomere length was measured by reference to molecular weight markers (SmartLadder, Eurogentec).

generations and the rate of telomere shortening at the *adh4:URA3* telomeres were measured in cell cultures established from different spores. The *est2Δ/rfa2Δ40* double mutant showed a growth reduction over generations comparable to that of the *est2Δ* single mutant, whereas growth reduction of the single *rfa2Δ40* mutant was less severe (**Fig. 3a**). We did not notice clear signs of senescence in *rfa2Δ40* cells, which can grow indefinitely, although at a low growth rate. In agreement with this observation, the short mean length seemed stable in long-term culture. However, after several streakings we noticed a high frequency of *rfa2Δ40* cells with recombined telomeres and higher growth rates. Thus, telomeres were not fully stable in *rfa2Δ40* cells.

We next quantified the rate of telomere shortening in growing cells harboring the same three mutation combinations. As expected, in cultures from wild-type spores, telomere length remained constant (data not shown); in cultures from spores lacking *EST2*, median telomere length decreased linearly at a rate of roughly 3 bp per generation (**Fig. 3b**). Notably, rates of telomere shortening were identical in cultures from *est2Δ* spores with and without the *rfa2Δ40* allele (**Fig. 3b**). Variation in initial telomere length between individual spore-derived cultures is likely to reflect differences in telomere length in the initial diploids or differences in the number of generations since the initial diploids. Initial length is not expected to influence the degradation rate[16]. We concluded that the *rfa2Δ40* allele does not increase the rate of telomeric DNA degradation resulting from the loss of telomerase. Indeed, the rate of telomere shortening in the *rfa2Δ40* single mutant was 2.3–2.4 bp per generation (**Fig. 3b**), substantially lower than the 3 bp per generation in cells lacking *EST2*. This, together with the fact that *rfa2Δ40* cells show a reduction of the median telomere length rather than a total loss of telomeric DNA, indicates that telomerase action in the *rfa2Δ40* mutant is reduced but not abolished. Overall, we conclude that *RFA2* is important for telomerase action.

The results described above prompted us to analyze the genetic interactions between *RFA2* and the ATM homolog *TEL1*, which has been proposed to regulate telomerase activity[23]. We first sporulated and dissected a diploid *RFA2/rfa2Δ40*; *TEL1/tel1; ADH4/TELadh4:URA3* strain. After two streakings starting from a spore and an overnight culture, we compared telomere lengths in isogenic *tel1/rfa2Δ40*, *tel1* and *rfa2Δ40* mutants for both the genetically marked *URA3* telomeres and telomeres carrying a conserved *Xho*I site in their Y' element (**Fig. 3c**). Although the sizes of the natural telomeres were similar in *rfa2Δ40* and *tel1* mutants, the *URA3*-marked telomeres were reproducibly shorter in *rfa2Δ40* cells than in *tel1* cells (by about 110 bp). Such differences between Y' telomeres and genetically marked telomeres have been reported elsewhere[24]. Most notably, the genetically marked and natural telomeres in *rfa2Δ40* and *rfa2Δ40 tel1* cells were almost identical in size (≈10 bp difference; **Fig. 3c**). We concluded that deleting *TEL1* does not exacerbate the telomere shortening in *rfa2Δ40* mutant cells.

Telomeric binding of RPA in wild-type and *rfa2Δ40* cells

We investigated whether the binding of Rfa1p and Rfa2p to telomeres was altered in *rfa2Δ40* cells. We carried out ChIP experiments at different stages of the cell cycle in *RFA1-MYC* and *RFA1-MYC/rfa2Δ40* cells, using polyclonal antibodies to Rfa2 and monoclonal antibodies to Myc

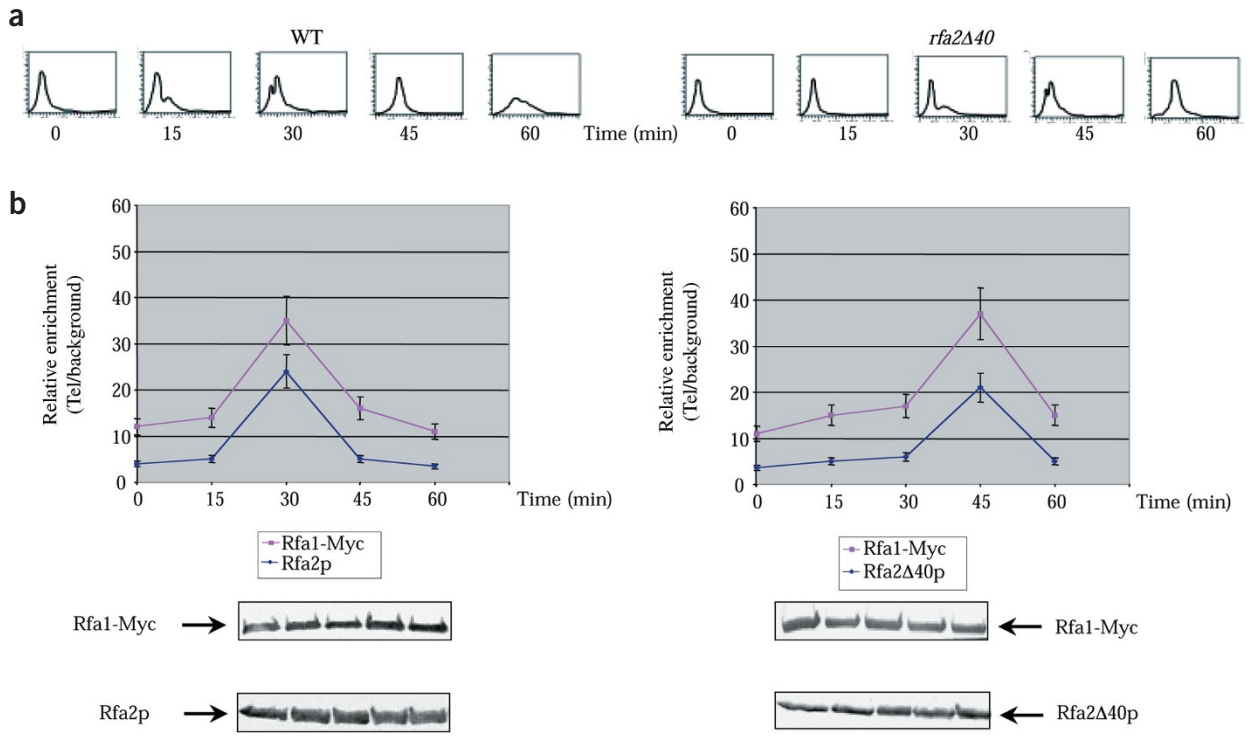

Figure 4 Telomeric association of Rfa2p (or Rfa2Δ40p) and Rfa1p in wild-type (WT) and *rfa2Δ40* cells. (**a**) FACS analysis of the synchronized UC1001 cells after release from α-factor-induced G1 block. (**b**) Amounts of telomeric DNA (pUra2 or Tel) determined by ChIP assays of chromatin from cells harvested every 15 min after release from an α-factor block. The amount of DNA was calculated by quantitative real-time PCR. Relative enrichment of Rfa2p- and Rfa1-Myc-bound telomeric DNA (pUra2/Tel) over background (pBdf1) is plotted against time. Amounts of Rfa2p, Rfa2Δ40p or Rfa1p-Myc immunoprecipitated protein at each time point are shown.

to monitor the binding of Rfa2p and a functional Rfa1-Myc fusion, respectively (**Fig. 4**). In the course of the cell cycle, the truncated version of Rfa2p bound to telomeres much like full-length Rfa2p. Like Rfa2p, Rfa1p bound to telomeres with a maximum in late S phase, and this was true in the *rfa2Δ40* strain as well as in the wild type (**Fig. 4**). Thus, *rfa2Δ40* does not alter RPA binding to telomeres. We also found that Rfa2p was coimmunoprecipitated with Rfa1p-Myc and vice versa, and that Rfa2Δ40p was also coimmunoprecipitated with Rfa1p-Myc and vice versa (data not shown). We therefore concluded that Rfa1p and Rfa2p both bind to telomeres with similar kinetics and that truncating the N terminus of Rfa2p does not affect the telomeric binding of Rfa2p and Rfa1p. Consistent with these results, Rfa2Δ40p still interacts with Rfa1p. Taken together, these data indicate that the role of RPA in telomere maintenance is most likely to involve the whole RPA heterotrimer. It is likely that an activity of RPA other than DNA-binding activity is responsible for the telomeric phenotype shown by *rfa2Δ40* mutant cells.

Binding levels of Rfa1-Myc (anti-Myc ChIP) and Rfa2p (anti-Rfa2p ChIP) to telomeric DNA in G1 phase was five and ten times, respectively, that for the nontelomeric genomic control (**Fig. 4**). By comparison, under the same ChIP conditions, telomeric binding of Myc-tagged Est1p, Cdc13p and Est2p in G1 phase was 4, 4.6 and 36 times, respectively, that of the control (**Fig. 5**). We therefore believe that the amount of RPA bound to telomeres in G1 phase is functionally important though low. The difference in the telomeric binding of Rfa2p and Rfa1-Myc may reflect a slightly higher background binding when antibodies to Rfa2p, as compared to antibodies to Myc, are used.

Rfa2p is required for telomeric binding of Est1p during S phase

We next assessed, using ChIP assays, whether the binding of Cdc13p, Est1p and Est2p to telomeric DNA is impaired in *rfa2Δ40* cells. A Myc-tagged version of *CDC13, EST1* or *EST2* was introduced into wild-type and *rfa2Δ40* strains bearing the *URA3*-marked telomere. Overall, the tagged proteins were functional. The tagged strains were synchronized in G1 phase with α-factor and released into the cell cycle. We monitored cell-cycle progression of both the wild-type and *rfa2Δ40* strains by FACS analysis, collecting samples every 15 min for ChIP assays. The amounts of telomeric DNA bound to Cdc13p and Est2p did not differ notably between the strains (**Fig. 5**). In both strains, telomeric binding of Cdc13p and Est2p increased in S phase (**Fig. 5**), although a proportion of Est2p is already associated with the telomeres in G1 phase (ref. 18). By contrast, the increase in telomeric binding of Est1p that normally occurs in S phase was severely impaired in the *rfa2Δ40* cells (**Fig. 5**). We concluded from these data that the *rfa2Δ40* mutation results in a specific defect in Est1-Myc telomeric binding in S phase.

To assess whether the Est1p telomeric binding defect observed in *rfa2Δ40* cells would not simply result from a shortened or perturbed

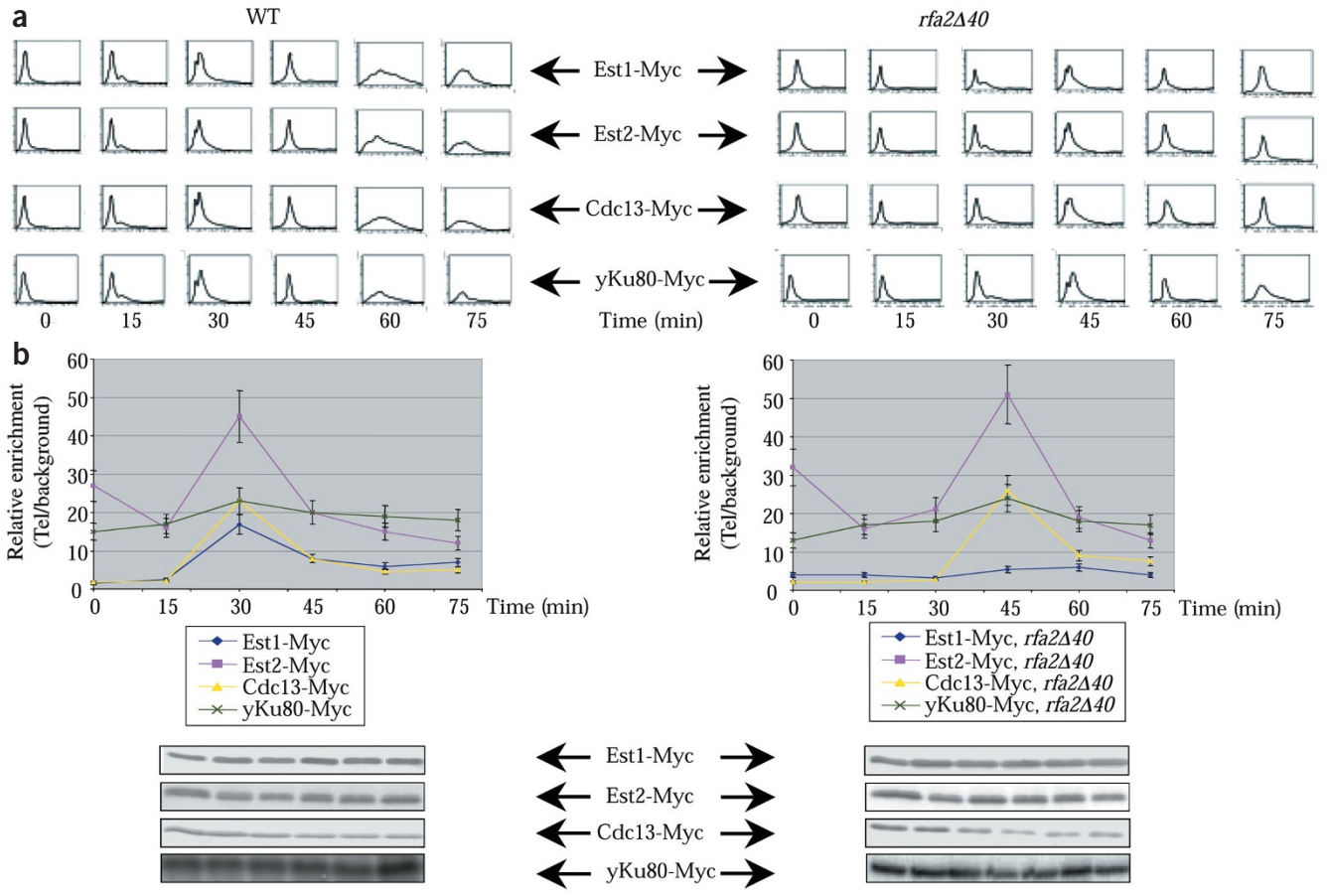

Figure 5 The *rfa2Δ40* allele impairs Est1p telomeric DNA binding during S phase. (**a**) FACS analysis of the indicated UC1001 cells after release from α-factor-induced G1 block. (**b**) Telomeric DNA binding of Est1-myc, Est2-myc, Cdc13-myc and yKu80-myc was monitored by ChIP assays (anti-Myc (9E10)) as described in **Figure 4**. Relative enrichment of Est1-myc, Est2-myc, Cdc13-myc and yKu80-myc-bound telomeric DNA over background is plotted against time. Immunoprecipitated proteins at each time point were analyzed by western blotting with anti-Myc (9E10).

telomere, we analyzed the telomeric binding of yKu80p by the same method using a yKu80-Myc fusion. A perturbation of yKu at the telomere might be predicted to lead to a loss of Est1p recruitment[25]. Similar yKu80p binding occurred in the wild type and the *rfa2Δ40* mutant. In both strains, yKu80p bound to telomeres throughout the cell cycle, with a slight increase in S phase (**Fig. 5**). Thus, the defect in Est1p recruitment in *rfa2Δ40* cells does not seem to be related to a perturbation of yKu80p localization to the telomere. This additive control supports a direct role of RPA in Est1p access to telomeres.

Following the same logic, we next analyzed whether the binding of Cdc13p, Est1p and Est2p would be affected in a *yku70Δ* mutant that has short telomeres (**Fig. 6**). We observed a slight decrease in the binding of Est2p to mutant telomeres in G1 and in S phase in the *yku70Δ* mutant, whereas binding of Cdc13p to telomeres in S phase was increased. Binding of Est1p to telomeres in S phase is slightly decreased in the *yku70Δ* mutant, whereas binding of Est1p to telomeres in S phase is severely decreased in the *rfa2Δ40* mutant (compare **Fig. 5b**, wild-type and *rfa2Δ40*, with **Fig. 6b**). From these results, we believe that the decrease of Est1p binding to telomeres is not an indirect consequence of telomere shortening but rather results from a direct role of RPA in enabling access of Est1p to telomeres.

rfa2Δ40 telomere length when Est1p is tethered to the telomere

That *rfa2Δ40* cells show a strong defect in the telomerase pathway of telomere length regulation and an altered binding of Est1p to telomeres indicates that RPA may facilitate loading of Est1p to promote telomerase action at telomeres. If this notion is correct, one can anticipate that the *rfa2Δ40* mutation would be suppressed if telomeric binding of Est1p were forced by expression of a hybrid protein consisting of Est1p and the DNA-binding domain of Cdc13p. We transformed wild-type and *rfa2*Δ40 cells with the plasmid pVL1120, which directs the expression of such a hybrid protein, Est1p-DBD_{CDC13} (ref. 14). Efficient telomere lengthening occurred in the *rfa2Δ40* mutant (>200 bp) (**Fig. 7**), and the growth defect of *rfa2Δ40* cells was suppressed by the transformation with pVL1120 (data not shown). This occurred for every clone we analyzed, although the extent of telomere elongation varied between transformants. These results confirmed that Cdc13p retains the ability to bind to telomeric DNA in *rfa2Δ40* mutants (as discussed above) and that the telomere length defect associated with the *rfa2Δ40* mutation can be corrected by forcing the loading of Est1p onto the telomere.

DISCUSSION

We have shown that RPA is present at telomeres and that truncation of the N-terminal region of Rfa2p greatly reduces telomere length. Because RPA participates in most aspects of DNA metabolism as a single-stranded DNA-binding protein, binding of RPA to telomeres might seem to be related to the presence of single-stranded telomeric DNA (G-tail). Consistent with this hypothesis, during the S phase of the cell cycle there is a general coincidence between the maximal telomeric binding of RPA described here, G-tail formation and telomere elongation. Based on all these results, we propose that RPA facilitates telomerase action during S phase.

Our ChIP analysis showed no notable differences between wild-type and *rfa2Δ40* cells in the amount of Cdc13p and Est2p associated with telomeres. In contrast, binding of Est1p in S phase is severely impaired in *rfa2Δ40* cells. Although the question was not directly addressed in this study, it is likely that Cdc13p is also required for proper Est1p loading during telomerase activation[14,15,18]. This suggests that telomere shortening in the *rfa2Δ40* mutant is not due to reduced binding of Cdc13p and Est2p to telomeric DNA, but rather reflects a decrease in the binding of Est1p to telomeres. This theory is in agreement with the fact that artificial tethering of Est1p to telomeres (via a Est1p-DBD_{CDC13} fusion protein) corrected the *rfa2Δ40*-associated defects. The binding of Est2p to telomeres seems to be independent of proper loading of Est1p. This is consistent with the idea that the role of Est1p is not to recruit Est2p to telomeres but

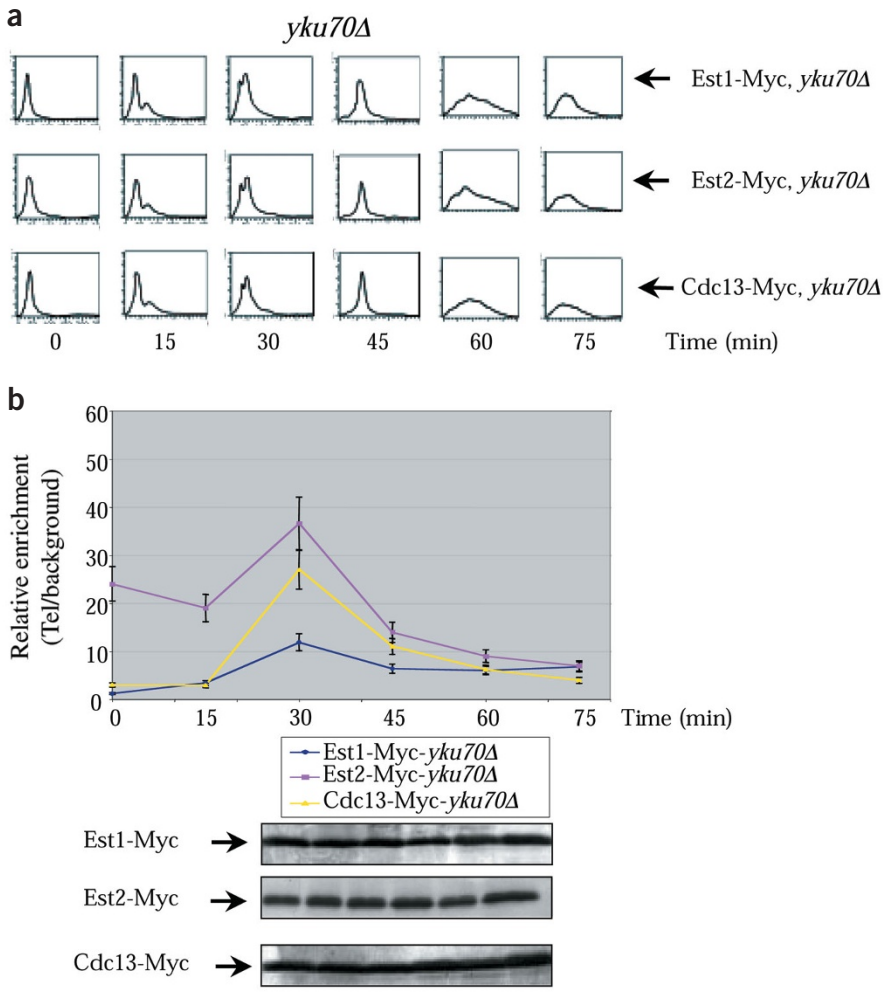

Figure 6 Association of Est1-myc, Est2-myc and Cdc13-myc in *yKu70Δ* cells. (**a**) FACS analysis of the indicated UC1001 cells after release from α-factor-induced G1 block. (**b**) Telomeric DNA binding of Est1-myc, Est2-myc and Cdc13-myc was monitored by ChIP assays as described in **Figure 5**. The wild-type control experiment is illustrated in **Figure 5b**.

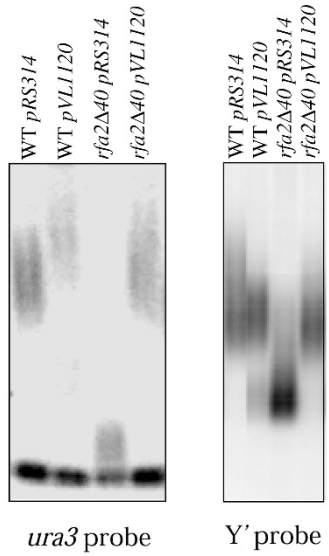

Figure 7 Telomere lengthening by targeted Est1p in *rfa2Δ40* cells. Telomere length was measured by Southern blotting using either *ura3* (left) or Y′ probe (right). pVL1120, which directs the expression of Est1-DBDCDC13 (ref. 14), derives from pRS314. Telomere length was measured by reference to molecular weight markers (SmartLadder, Eurogentec). WT, wild type.

rather to activate an already bound form of telomerase[18]. We have been unable to coimmunoprecipitate Est1p (or Cdc13p or Est2p) with Rfa2p (data not shown), indicating that control of telomerase activity by Rfa2p may not be mediated by a stable interaction between Rfa2p and Est1p. Either the putative interaction between RPA and Est1p is too infrequent, too transient or both to be detected, or RPA does not directly recruit Est1p but rather maintains single-stranded DNA in a state amenable to Est1p binding. For instance, RPA could act through the stimulation of a helicase activity, opening up the duplex portion of the telomeric DNA and thereby facilitating the binding of Est1p. Overall, we propose that RPA and Cdc13p cooperate to allow Est1p binding to telomeres and subsequently promote telomerase action (**Fig. 8**). The *rfa2Δ40* allele seems to be specifically impaired in the Est1p loading step (**Fig. 8**).

Our results do not rule out roles for RPA in other aspects of telomere length regulation, including the control of telomeric DNA degradation and the coupling of telomerase activity to lagging-strand synthesis[17,26]. RPA has consistently been shown to interact with the DNA polymerase α–primase complex[27]. In addition, we found that the *rfa1-M2* allele[28] of the large subunit of RPA (*RFA1*) results in telomere elongation (data not shown), indicating a possible role of RPA in $C_{1-3}A$ strand synthesis by DNA polymerase α.

We have found that deleting *TEL1* in *rfa2Δ40* cells does not aggravate telomere shortening, indicating that RPA and Tel1p may act in the same pathway. Notably, the phenotypes of *rfa2Δ40* cells show similarities with the telomeric phenotypes of cells devoid of Mec1p and Tel1p: (i) *rfa2Δ40* cells and cells lacking Tel1p and Mec1p have residual telomerase activity (as described here and in refs. 29,30); (ii) the association of Cdc13p with telomeres occurs efficiently in *tel1Δ* cells and *rfa2Δ40* cells (described here and in ref. 31); and (iii) forcing the Cdc13p-dependent recruitment of telomerase to the telomeres suppresses the growth defects of both *rfa2Δ40* and *mec1 tel1* cells (described here and in ref. 31). Another link between Rfa2p, Mec1p and Tel1p is provided by the fact that Rfa2p is phosphorylated during S phase mainly by Mec1p but also by Tel1p (ref. 5). We can speculate from these data that RPA could be one of the targets of these kinases at telomeres, although a very recent report indicates that substituting alanine residues for the two serines (Ser122 and Ser238) of Rfa2p that are in SQ motifs (preferred targets of the ATM kinase family) does not affect telomere length[32]. Notably, the same study showed that the *rfa2-55* allele (encoding E90A and D91A substitutions produces short telomeres[32,33].

The control of telomerase activity by two single-stranded DNA–binding proteins, Cdc13p and RPA, may provide a means to couple telomerase activity tightly to the formation of 3′ overhangs during telomere replication. Thus, RPA might be a crucial link between telomere homeostasis and the replication of chromosome ends. The role of RPA in the loading of Est1p at telomeres may be evolutionarily conserved, as both RPA and Est1p are present in human as well as yeast cells[2,34,35].

METHODS

Mutant strain construction and telomere experiments. A deletion removing the *RFA2* intron and the region extending from codon 3 to codon 40 of *RFA2* (*rfa2Δ40*) was introduced as described[8]. By replacing the chromosomally encoded *RFA2* with the mutated *rfa2Δ40*t allele, we generated an allele (*rfa2-165*) encoding a truncated protein extending from residue 1 to residue 165 that is coexpressed with Rfa2pΔ40. To get rid of potential effects of *rfa2-165*, this inviable, truncated allele was fully disrupted with a *kanMX4* marker[8].

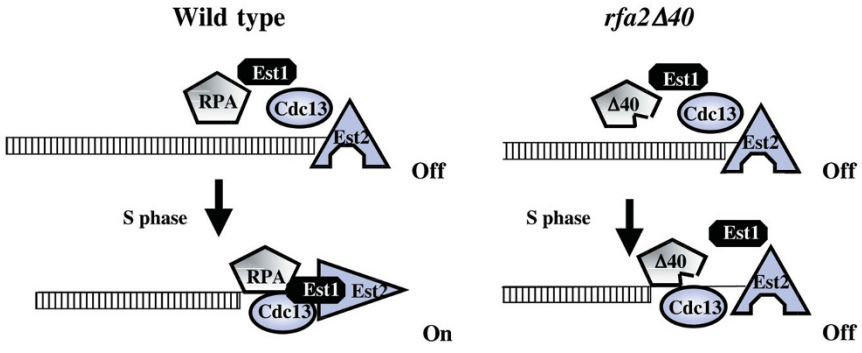

Figure 8 Model of telomerase action during the cell cycle. Left, in G1 phase, a proportion of the catalytic subunit of telomerase (Est2p) is bound at the telomere in an inactive form[18]. In late S phase, the 3′ overhang lengthens[12] and RPA, Est1p and Cdc13p become bound to the telomere[18,19] (see text). Binding of Est1p depends on both Cdc13p[14,15,18] and RPA, whereas binding of RPA and Cdc13p can occur independently (see text). The complex formed between the 3′ overhang, RPA, Cdc13p and Est1p is required for telomerase action at the chromosome end. The interactions between RPA, Est1p, Est2p and Cdc13p are likely to be transient, indirect or both, as the complex has not been identified in coimmunoprecipitation experiments. Notably, in G1, we found a basal level of RPA binding to telomeres (not shown in this model). Right, in the presence of the *rfa2Δ40* mutation, the RPA complex (Δ40) binds to the 3′ overhang but is unable to load Est1p. Consequently, the telomerase stays in its inactive form despite the proper fixation of Cdc13p. Because residual telomerase activity can be detected in *rfa2Δ40* cells, one can imagine that a weak loading of Est1p can still occur.

Rfa2p was tagged at its C terminus with 18 MYC epitope with pGG1 (ref. 8). Epitope-tagged Cdc13p, Est1p and Est2p were constructed to retain their endogenous promoters. Cdc13p and Est2p were 18 Myc epitope–tagged in their N-terminal portions using pVL1042 and pVL1001 linearized plasmids (kindly provided by V. Lundblad). Est1p was 13 Myc epitope–tagged in its C-terminal portion using one-step PCR. The functional *RFA1-MYC18* allele was obtained from T. Tanaka[36]. We carried out silencing assays, telomere length analysis by Southern blotting, G-strand extension at telomeres and synchronization as described[8,16,21]. We carried out most of the experiments in yeast strains isogenic to UCC1001 (*MATa ade2-101 his3Δ200 leu2Δ1 trp1Δ1 ura3-52 TELadh4:URA3*).

We combined the *rfa2Δ40* mutation with *est2Δ* as follows: we crossed the yeast strain JKM179 *MATα TELadh4:URA3 est2Δ* (grown for 25 generations after the disruption of *EST2*) to JKM139 *MATa rfa2Δ40* to produce the diploid JKM179/JKM139 *(MATα /MATa EST2/est2Δ RFA2/rfa2Δ40 ADH4/TELadh4:URA3 Δho/Δho Δhml:ADE1/Δhml:ADE1 Δhmr/Δhmr ade1-100/ade1-100 leu2-3,112/leu2-3,112 lys5/lys5 trp1:hisG/trp1:hisG ura3-52/ura3-52 ade3:GAL:HO/ade3:GAL:HO*). Integration of a PCR product containing the nourseothricin-resistance (*NAT*) gene amplified from pAG25 (a kind gift from P. Goldstein and J. McCusker) disrupted *EST2*. We streaked the diploid strain several times in rich medium before sporulation, then identified spores with the appropriate genotypes and determined their telomere lengths as described[16].

Southern blotting. For Southern blots, *Eco*RV- and *Hin*dIII-digested genomic DNAs were resolved in 1.2% agarose gel and transferred to Hybond-N+ membrane (Amersham Biosciences). We probed the digested genomic DNAs with a radiolabeled *URA3* DNA fragment. For Y' Southern blots, *Xho*I-digested genomic DNAs were resolved in 1.2% agarose gel and transferred to Hybond-N+ membrane and probed with a radiolabeled Y' DNA fragment.

Chromatin immunoprecipitations assays. We carried out ChIP assays as described[8] in yeast strains isogenic to UCC1001. Immunoprecipitation of cross-linked DNA was done with agarose-conjugated 9E10 monoclonal antibodies (to Myc; Santa Cruz Biotechnology) or with polyclonal antibodies to Rfa2p (kindly provided by S. Brill, Rutgers University, Piscataway, New Jersey and B. Stillman, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, respectively). The results obtained with anti-Rfa2p antibodies respect to the telomeric binding of Rfa2p are similar to those described obtained with the *RFA2-MYC* strain with anti-Myc 9E10 antibodies. We used primer pairs specific for the fragmented *URA3* to amplify the 400-bp pUra2 and primer pairs specific for the telomeric TG_{1-3} repeats and *URA3* to amplify the 230-bp pUra1. The chromosome VI-R telomere-specific primers (SG355 and SG356), which have been described previously[37], produced a 400-bp fragment located 0.6 kb from the TG repeat. Control primers specific for the *BDF1* control sequence produced a 100-bp PCR fragment. (All primer sequences are available on request.) We calculated the amounts of telomeric and nontelomeric DNAs in input and immunoprecipitated samples by quantitative PCR with a LightCycler PCR (Roche) as described[8]. Relative enrichment of telomeric DNA (pUra2) over background (pBdf1) in the immunoprecipitates was calculated as follows: $(IP_a$ pUra2/IP_a pBdf1) ($input_a$ pBdf1/$input_a$ pUra2); IP_a and $input_a$ represent the amount of PCR product in the immunoprecipitates and the input samples, respectively.

Yeast one-hybrid assay for telomere-interacting proteins. Yeast strains were derived from YM701 (*MATa ura3-52 his3-200 ade2-101 lys2-801 trp1-901 tyr1*). Strains *HIS-Int-CA* and *HIS-Tel* and plasmids pJG4-5/*RIF2* and pJG4-5/*CDC13N* were kindly provided by V. Zakian[20]. We cloned *RFA2* into pJG4-5 as an *Xho*I DNA fragment. To monitor telomere interaction, we assayed cells on 3% Gal –His test plates containing 5 mM 3-amino-1,2,4-triazole (3-AT). Control plates were 3% Gal –Trp. Plates were incubated at 30 °C for 5 d.

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COMPETING INTERESTS

The authors declare that they have no competing financial interests.

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