Two Pathways Recruit Telomerase to *Saccharomyces cerevisiae* Telomeres

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**Abstract**

The catalytic subunit of yeast telomerase, Est2p, is a telomere associated throughout most of the cell cycle, while the Est1p subunit binds only in late S/G2 phase, the time of telomerase action. Est2p binding in G1/early S phase requires a specific interaction between telomerase RNA (TLC1) and Ku80p. Here, we show that in four telomerase-deficient strains (cdc13-2, est1A, tlc1-SD, and tlc1-BD), Est2p telomere binding was normal in G1/early S phase but reduced to about 40–50% of wild type levels in late S/G2 phase. Est1p telomere association was low in all four strains. Wild type levels of Est2p telomere binding in late S/G2 phase was Est1p-dependent and required that Est1p be both telomere-bound and associated with a stem-bulge region in TLC1 RNA. In three telomerase-deficient strains in which Est1p is not Est2p-associated (tlc1-SD, tlc1-BD, and est2A), Est1p was present at normal levels but its telomere binding was very low. When the G1/early S phase and the late S/G2 phase telomerase recruitment pathways were both disrupted, neither Est2p nor Est1p was telomere-associated. We conclude that reduced levels of Est2p and low Est1p telomere binding in late S/G2 phase correlated with an est phenotype, while a WT level of Est2p binding in G1 was not sufficient to maintain telomeres. In addition, even though Cdc13p and Est1p interact by two hybrid, biochemical and genetic criteria, this interaction did not occur unless Est1p was Est2p-associated, suggesting that Est1p comes to the telomere only as part of the holoenzyme. Finally, the G1 and late S/G2 phase pathways for telomerase recruitment are distinct and are likely the only ones that bring telomerase to telomeres in wild-type cells.

**Introduction**

Telomerase is a specialized reverse transcriptase that lengthens the 3' end of telomeric DNA. In *Saccharomyces cerevisiae*, the template for telomere elongation is a short stretch within the 1158 base TLC1 telomerase RNA. Est2p is the *S. cerevisiae* telomerase catalytic subunit, while Est1p and Est3p are two telomerase subunits whose roles in telomerase action are less well understood. Although Est2p and TLC1 RNA are sufficient for telomerase catalytic activity in vitro, all three EST proteins, as well as TLC1 RNA are required in vivo. Telomerase deficient strains such as *tlc1Δ, est2Δ, est1Δ*, and *est3Δ* are viable but slowly lose telomeric DNA (reviewed in [1]). After 50–100 generations, when telomeres are very short, chromosome loss increases in these strains, and most cells in the population die, a collection of behaviors known as the ever shorter telomere (est) phenotype [2].

The key events in *S. cerevisiae* telomere replication and processing occur in late S/G2 phase. Most of the ~300 bp yeast telomere is replicated by semi-conservative DNA replication, which occurs very late in S phase. After semi-conservative replication, C-strand resection generates ~50–100 base G-tails at both ends of DNA molecules. These G-tails are repaired by C-strand resynthesis prior to mitosis [3–5]. Telomerase lengthening of telomeres also occurs late in the cell cycle [6,7].

Cdc13p is a single-strand TG1–3 sequence specific DNA binding protein [8,9] that associates in vivo with the G-tails that constitute the very ends of yeast chromosomes [10,11]. Although the Cdc13p complex has an essential role in protecting telomeres from degradation [12–14], there are also alleles of *CDC13*, such as *cdc13-2*, that have normal end protection activity but confer an est phenotype [9]. Cdc13p and Est1p interact by two-hybrid, co-immuno-precipitation [15], and genetic criteria [16]. Moreover, fusions between the DNA binding domain of Cdc13p and Est2p can maintain telomeres in the absence of Est1p [17]. Together these data suggest that Est1p acts by recruiting Est2p to the telomere in late S/G2 phase. This recruitment is thought to occur via a specific interaction between Est1p and Cdc13p that is lost in telomerase defective *cdc13-2* cells. The association of Est3p with Est2p is Est1p-dependent [18].

In previous work, we used chromatin immuno-precipitation (ChIP) to test different aspects of this recruitment model [19]. Consistent with the model, Est1p binding to telomeres is limited to late S/G2 phase, and Cdc13p binding, which occurs throughout the cell cycle, increases enormously at this time, concomitant with the appearance of long G-tails. However, Est2p is telomere associated throughout most of the cell cycle, not just in late S/G2 phase as the model predicts. The high Est2p binding in late S/G2 phase is reduced by ~50% in the telomerase deficient *cdc13-2* strain, while Est2p binding earlier in the cell cycle is unaffected. The telomere association of Est2p at times when telomerase is not active is also inferred by fluorescent in situ hybridization, which shows colocalization of TLC1 telomerase RNA with telomeres in G1 and S

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Author Summary

Duplication of linear DNA is complicated by the fact that conventional DNA polymerases cannot copy their ends. From yeasts to humans, replication of DNA ends, called telomeres, is accomplished by a telomere-dedicated reverse transcriptase called telomerase that uses its RNA subunit as a template. We show that there are two genetically distinct pathways that recruit yeast telomerase, Est2p, to telomeres in a cell cycle–dependent manner. Only one of the two pathways, the pathway operating late in the cell cycle, is required for telomere maintenance. In telomerase-deficient strains, the amount of Est2p that is telomere-bound late in the cell cycle is about 50% of wild type levels. Maintenance of functional levels of Est2p late in the cell cycle requires that Est1p, another telomere subunit, be telomere-bound. In addition, Est1p must be associated with Est2p via an interaction between it and telomerase RNA. Human telomerase is not active in most somatic cells, but is critical for stem cell longevity. Even a modest reduction in telomerase has a serious impact on human health. The sensitivity of yeast to reduced levels of telomerase-associated telomerase may help us understand why human stem cells require high levels of telomerase for their survival.

Results

Est2p Telomere Binding in Late S/G2 Phase (but not G1 Phase) Is Reduced in est1A Cells

Chromatin immuno-precipitation (ChIP) was used to determine the telomere association of proteins involved in telomere maintenance in WT and mutant cells. We used strains in which the protein being studied was multiply epitope tagged at its endogenous locus and was the only form of the protein in the cell. Functionality of epitope tagged proteins was determined by their effects on telomere length and other telomere phenotypes. In earlier experiments, we used a Myc-tagged Est2p that was not fully functional as telomeres were ~50 bps shorter in its presence [19]. When this Myc-tagged Est2p was introduced into an est1A strain, cells senesced so rapidly that it was not possible to determine whether Est2p binds telomeres in est1A cells.

The function of epitope tagged Est2p was improved by inserting a flexible linker of eight glycine residues between the carboxyl terminus of Est2p and multiple Myc epitopes (hereafter called Est2-G8-Myc) [21,25]. This allele supports WT telomere length, does not senesce when combined with deletion of 1KL, and est1A cells carrying this Est2-G8-Myc can be grown for 50 to 100 cell divisions before they senesce. This Est2-G8-Myc allele was used to determine if Est2p telomere binding is Est1p-dependent.

Otherwise isogenic WT or est1A cells expressing Est2-G8-Myc were arrested in late G1 phase with alpha factor (0 minute time point) and then released into the cell cycle at 24°C. Samples were taken at 15 min time intervals and processed for FACS to determine position in the cell cycle, and by ChIP to determine Est2p association with telomeric DNA. Under these conditions, cells were in G1 phase at 0 and 15 min, in S phase at 30, 45, and 60 minutes, and in G2/mitosis at 75 and 90 min [19,21] (and data not shown). None of the mutations or epitope tagged proteins had reproducible effects on cell cycle progression [19,21] (and data not shown). ChIP samples were analyzed by quantitative multiplex PCR using primer pairs specific for the modified VII-L telomere (TEL), sub-telomeric VII-L DNA (ADH), or a sequence far from a telomere (ARO) (Figure 1A, B, left). Alternatively we examined association with the native VI-R telomere and ARO (Figure 1A, B right). For all synchronies, a representative gel from one of the three or more independent synchronies is shown. Fold enrichment is binding at telomere VII-L or VI-R relative to binding at ARO and normalized to input DNA [21]. Graphs are the compiled data from the three or more independent synchronies for a given strain; error bars are one standard deviation from the average for each time point.

As shown previously [19,21], in WT cells Est2p had high telomere association in G1 and early S phase (0 through 30 min), a modest decline in mid-S phase, a second peak in late S/G2 phase (60 minutes), and then a decline in association as cells progressed to the end of the cell cycle (Figure 1A, white squares). Est2-G8-Myc association with the VII-L telomere in est1A cells was similar to what was seen in WT cells except that binding in late S/G2 phase (60–90 min) was significantly reduced (Figure 1A left, black circles; see figure legends for P values). A similar pattern of Est2-G8-Myc binding in the absence of Est1p was seen at telomere VI-R (Figure 1A right). This pattern of binding was very similar to that seen for Est2-G8-Myc binding in another telomerase defective strain, cdc13-2 (Figure 1B, black triangles). The results for cdc13-2 presented here with Est2-G8-Myc are similar to our published data carried out with the less functional Est2p-Myc and using different quantitation methods [19]. Reduced Est2p binding in est1A and cdc13-2 cells was not due to reduced abundance of Est2p (Figure 1C, lanes labeled est1A and cdc13-2). We conclude that Est1p is not essential for Est2p telomere binding but is required for WT levels of Est2p binding in late S/G2 phase.

Est1p Telomere Binding Is Low in cdc13-2 Cells

In addition to changing our tagging strategy, a series of technical changes were made in the ChIP protocol that increased
Figure 1. Est2p telomere binding in late S/G2 phase is reduced in est1Δ and cdc13-2 cells. Cells expressing Est2-G8-Myc or lacking a Myc-tagged protein (no tag) were arrested in G1 phase. After release from the arrest, cells were grown at 24°C and samples taken at 15 min intervals for FACS and chromatin immuno-precipitation (ChIP). After DNA purification, PCR amplification was carried out with telomeric (TEL), subtelomeric (ADH), and non-telomeric (ARO) primers on DNA from immuno-precipitates (IP) or whole cell lysates (Input). Two-fold serial dilutions of input DNA established the linear range of the reactions (Input, top right). In this and subsequent figures, the agarose gels are representative data from mutant strains. For each time point, binding is expressed as the relative fold enrichment of TEL over ARO signal after normalization to input DNA. Error bars are ±1 standard deviations from ≥3 independent synchronies. A. Est2p binding to VII-L (left) and VI-R (right) telomeres in synchronous est1Δ (black circles) versus WT (white squares), or untagged (white triangles) cells. The values for Est2p binding to the VII-L telomere were not significantly different in WT versus est1Δ cells (P-values >0.05), except at 60 (P = 0.002), 75 (P = 0.02) and 90 (P = 0.04) minutes. Est2p binding to the VI-R telomere in WT versus est1Δ cells was significantly different only at 60 (P = 0.01) and 75 (P = 0.02) minutes. B. Est2p binding to VII-L (left) and VI-R (right) telomeres in synchronous cdc13-2 (black triangles) versus WT (white squares), or untagged (white triangles) cells. Est2p binding to the VII-L telomere in WT versus cdc13-2 cells was significantly different only at 60 (P = 0.01) and 75 (P = 0.02) min. At VI-R, binding was significantly different only at 75 minutes (P = 0.005) C. Western analyses of Est2p-G8-Myc or α-tubulin levels in extracts from WT and mutant strains with duplicate extracts prepared from independent colonies. The lanes labeled Δ48 are from tlc1Δ48 cells; 13-2 is cdc13-2; DM is double mutant tlc1Δ48 cdc13-2. Est2p-G8-Myc in lane 3 (tlc1Δ) was detectable upon longer exposure; see last lane that has protein sample from another tlc1Δ isolate and Figure 4C.
the signal to noise ratio in these experiments [21]. These changes were particularly important for Est1p since in our earlier work, the association of Est1p with telomeres in late S phase was enriched only five fold over background [19]. Under the conditions of these previous experiments, Est1p bound equally well to telomeres in WT and cdc13-2 cells. Since the result was surprising (see introduction), we reid this experiment using our more sensitive ChiP methods.

As shown previously [19], in WT cells, Est1p binding to the VII-L telomere peaked in late S phase (60 min) (Figure 2A, left, white boxes). With the improved ChiP protocols, peak Est1p binding was 20-fold above background. Contrary to our previous results, Est1p telomere binding to the VII-L telomere in cdc13-2 cells was much lower than WT, only ~4 fold above background (Figure 2A, left, black triangles). Although this binding was low, it was significantly higher than the signal with the no-tag control strain at all time points (Figure 2A). Reduced but significant Est1p binding was also seen at telomeres VI-R (Figure 2A, right) and XV-L (data not shown) in cdc13-2 cells. Western analysis demonstrated that this reduced Est1p telomere binding was not due to an effect of the cdc13-2 mutation on Est1p levels (Figure 2B, compare WT lanes to lanes labeled 15-2). We attribute the difference in these results compared to our earlier studies to the increased sensitivity of the current ChiP assay.

**Est1p Telomere Binding Is Est2p-Dependent**

By multiple criteria, Est1p interacts with Cdc13p, and this interaction is thought to recruit Est2p to telomeres (see introduction). If Est1p binding depends solely on its ability to interact with Cdc13p, Est1p might bind telomeres even in an est2A strain. However, using synchronous cells, Est1p-Myc binding to telomeres VII-L and VI-R in est2A cells (Figure 2C, black triangles) was very low, similar to background levels (Figure 2C, no tag, white triangles). The very low Est1p at est2A telomeres supports the interpretation that the signal for Est1p telomere binding in est2A-2 cells (Figure 2A) was real. The absence of Est1p binding was not due to reduced levels of Est1p in est2A cells (Figure 2B, compare WT to est2A lanes).

**Est1p Telomere Binding Is Low in the Absence of a Specific Interaction between Est1p and TLC1 RNA**

TLC1 RNA is immuno-precipitated with Est1p [26,27]. RNA structure analysis identified a potential stem-bulge region contained within nucleotides 600 to 669 that is conserved among different yeasts [24]. Both the putative 9 bp stem and the 5’ bulge region are essential for telomerase function in different yeasts [24]. Both the putative 9 bp stem and the 5’ bulge region are essential for telomerase function in different yeasts [24]. The stem of TLC1 RNA with the 5’ bulge are essential for telomerase function in different yeasts [24]. The stem of TLC1 RNA with the 5’ bulge are essential for telomerase function in different yeasts [24]. The loss of interaction with TLC1 RNA is specific for Est1p binding to telomeres (Figure 3). Est1p-Myc binding was 10.1 fold; VII-L, 12.2 fold; VI-R; lanes labeled SD) or tlc1-SD (3.5 fold, VII-L, 2.4 fold, VI-R; lanes labeled BD) alleles was not significantly different from binding in tlc1-1A cells (2.5 fold, VII-L, 3.0 fold, VI-R) (Figure 3B). In all three tlc1 alleles, Est1p binding was low but significantly higher than in the no-tag control. In contrast, the telomerase proficient tlc1-SC allele supported high levels of Est1p-Myc telomere binding, (9.9 fold, VII-L; 6.3 fold, VI-R; lanes labeled SC), a level that was not significantly different from WT. Low Est1p-Myc binding was not due to difficulties detecting proteins at short telomeres as Cdc13p-Myc telomere binding was high in all backgrounds (Figure 3D).

**Est2p Telomere Binding in Late S Phase Requires the Stem-Bulge Region of TLC1 RNA**

By co-immunoprecipitation, Est2p interacts normally with both TLC1-SD and TLC1-BD RNAs [24]. Although the late S/G2 peak of Est2-G8-Myc binding did not occur in est1A cells (Figure 1A), this effect could be due to an off-telomere effect of Est1p on Est2p structure or function that can not occur in the absence of Est1p. Alternatively, Est1p telomere binding may be required for Est2p binding. Since Est1p was present at normal levels (Figure 3C) but had low telomere association in tlc1-SD and tlc1-SC cells (Figure 3C, B), it is possible to distinguish between these possibilities in these strains.

In synchronous tlc1-SD cells, Est2-G8-Myc binding to the VII-L telomere was very similar to the pattern of Est2-G8-Myc binding in est1A and est1A-2 cells (compare Figure 4C, tlc1-SD, black diamonds, to Figure 1A, est1A, black circles and 1B, est1A-2, black triangles). That is, Est2-G8-Myc binding was at WT levels in G1 and early S phase but was significantly reduced in late S/G2 phase. Est2-G8-Myc binding was also determined in asynchronous cells expressing the TLC1 alleles (Figure 4B). As shown previously [24], Est2p binding was absolutely dependent on TLC1: Est2-G8-Myc binding was at background levels in tlc1A cells (enrichment of 1.3 fold, VII-L; 0.8 fold, VI-R). The high Est2-G8-Myc binding in WT cells (17.3 fold, VII-L, 12.2 fold VI-R) was significantly reduced but still detectable in asynchronous tlc1-1D cells (enrichment at VII-L, 8.0 fold; VI-R, 3.2 fold). The reduction in Est2-G8-Myc telomere binding in tlc1-1D and tlc1-1D cells was not due to a reproducible decrease in Est2p abundance (Figure 4C). These results demonstrate that Est1p is needed in cis (i.e., at the telomere) to support WT levels of Est2p telomere binding in late S/G2 phase.

**Neither Est2p nor Est1p Is Telomere-Associated when Both the TLC1-Ku80p G1 and the TLC1-Est1p Late S/G2 Recruitment Pathways Are Mutated**

Eliminating the specific interaction between Yku80p and TLC1 RNA with the tle1A48 or yku80-135i mutations [21,23] eliminates Est2p at the telomere in G1 and early S phase [21]. Mutations that
Figure 2. Est1p telomere binding is greatly reduced in synchronous cdc13-2 cells and eliminated in est2Δ cells.

Methods and symbols are as described in the Figure 1 legend except that cells expressed Est1-Myc.

A. Est1p binding to VII-L (left) and VI-R (right) telomeres in synchronous cdc13-2 (black triangles) versus WT (white squares), or untagged (white triangles) cells. Est1p binding to the VII-L telomere was significantly higher in cdc13-2 cells than in the no-tag control at times of peak Est1p binding (P values ranged from 0.0003 at 60 min to 0.035 at 90 min). Est1p binding to the VI-R telomere was significantly different from the no-tag control at all time points (P values ranged from 0.0006 at 60 min to 0.0065 at 90 min.).

B. Western analyses of Est1p-Myc versus α-tubulin levels in extracts from WT and mutant strains. The lanes labeled J48 are from different tlc1Δ colonies; J48 is cdc13-2; DM is double mutant tlc1ΔJ48 cdc13-2.

C. Est1p binding to VII-L (left) and VI-R (right) telomeres in synchronous est2Δ (black triangles) versus WT (white squares), or untagged (white triangles) cells. Est1p binding to the VII-L and VI-R telomeres in est2Δ cells was modestly higher than the no-tag control only at 45 (P = 0.04 VII-L; 0.013, VI-R) and 60 min (P = 0.04, VII-L; 0.03, VI-R).

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Figure 3. Est1p telomere binding is low in the absence of the stem-bulge region of TLC1 RNA. Methods and symbols are as described in legend of Figure 1 except that cells expressed Est1-Myc and for panels B–D, asynchronous log phase cells were analyzed. A. Est1p binding to VII-L telomere in synchronous tlc1-SD (black diamonds) versus WT (white squares), or untagged (white triangles) cells. Although Est1p binding from 30 through 90 min was higher in tlc1-SD cells than in the no tag control, the difference was significant (P = 0.045) only at 45 min. B. Est1p binding to VII-L (top) or VI-R (bottom) telomeres in WT and mutant asynchronous cells. Bar graphs show average Est1p association with telomere VII-L (dark grey) or VI-R (light grey) with error bars indicating ± one standard deviation from that average; abbreviations for strains are SD, tlc1-SD; SC, tlc1-SC; BD, tlc1-BD. The level of binding in tlc1-SC cells was not significantly different from WT (P = 0.16, VII-L; 0.07, VI-R). The level of binding in both tlc1-SD and tlc1-BD cells was not significantly different from tlc1Δ cells (P values ranged from 0.22 to 0.29). The level of Est1p binding in tlc1Δ cells was significantly higher than in the no-tag control (P = 0.011, VII-L; 0.029, VI-R). Likewise, the level of Est1p binding in tlc1-SD (P = 0.03, VII-L; 0.13, VI-R) and tlc1-BD (P = 0.074, VII-L; 0.07, VI-R) was mostly significantly higher than the no-tag control. C. Western analyses of Est1p-Myc in extracts from WT and mutant strains. Abbreviations for strains are same as in panel B. D. Cdc13p binding to VII-L (top) and VI-R (bottom) telomeres in asynchronous mutant and WT cells. Cdc13p binding was not significantly different in tlc1-SD versus tlc1-BD cells (P = 0.5, VII-L; 0.2, VI-R). Cdc13p binding was significantly higher than in WT at both telomeres in tlc1-SD (0.016, VII-L; 0.009, VI-R) and at VI-R in tlc1-BD (P = 0.015; but not at VII-L, P = 0.072). Cdc13p binding was similar in WT and tlc1Δ cells (P = 0.08, VII-L; 0.06, VI-R).

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strongly reduced the amount of telomere bound Est1p, such as cdc13-2, tlc1-SD and tlc1-BD, or an est1A allele that eliminates Est1p altogether, lack high levels of Est2p binding in late S/G2 phase [19] (Figure 1A, B; 4A). To determine if these pathways are the only ones that recruit telomerase to yeast telomeres, Est2p telomere binding was examined in double mutants that eliminate both pathways. Est2-G8-Myc binding was determined in synchronous tlc1-SD cdc13-2 cells (Figure 5A). Although Est2p abundance was normal in this background (Figure 1C, lane DM, double mutant), Est2-G8-Myc binding to the VII-L telomere (Figure 5A, black triangles) was not significantly different from the no tag control (Figure 5A, white triangles). Est2-G8-Myc binding was also very low at both the VII-L and VI-R telomeres in asynchronous yku80-135i tlc1-SD, yku80-135i tlc1-BD and tlc1-SD est1A cells (Figure 5B). Est1-Myc binding to both the VII-L and VI-R telomeres was indistinguishable from the no-tag control strain in synchronous tlc1-SD cdc13-2 cells (Figure 6). We conclude that the TCL1-Ku mediated pathway that recruits Est2p to telomeres in G1 phase [21] and the pathway that requires specific interactions of Est1p with both Cdc13p (Figure 2A) and TCL1 RNA (Figure 3A, B) that maintains high levels of telomere bound Est2p in late S/G2 phase are the only pathways that recruit Est2p to DNA ends in otherwise WT cells.

Discussion

A specific interaction between a 48 bp stem-loop region in TCL1 RNA and Yku80p brings Est2p to the telomere in G1 and early S phase [21]. This TCL1-Ku interaction is also required for WT levels of telomere-associated telomerase in late S/G2 phase as both Est2p (~40–50% of WT) and Est1p (~33% of WT) telomere binding are reduced in these backgrounds. Cells that lack the Ku-TLC1 RNA interaction (tlc1-SD, yku80-135i, and ykaA) have short but stable telomeres and do not senesce.

In contrast, four est strains examined here (est1A, cdc13-2, tlc1-SD, and tlc1-BD) as well as est3A cells (data to be published elsewhere), had WT levels of telomere associated Est2p in G1 and early S phase but reduced (~50% of WT) Est2p telomere binding in late S/G2 phase (Figure 1A, B; 4A). The only est strain (other than est2A) that did not have this pattern was tlc1A in which there was no Est2p telomere binding at all [19] (Figure 4B). Reduced Est2p telomere binding was not associated with a marked reduction in Est2p abundance except in the tlc1A strain [19] (Figure 1C, 4C).

Est2p binding in late S/G2 phase was Est1p dependent (Figure 1A). However, the presence of Est1p was not sufficient for WT Est2p telomere association as Est2p binding was equally reduced in late S/G2 phase in tlc1-SD and tlc1-BD cells (Figure 4A, B) where Est1p was present (Figure 3C), but is neither TCL1-associated [24] nor telomere-bound (Figure 3A, B). The fact that Est2p does not bind telomeres at all in a tlc1A strain [19] (Figure 4B) is consistent with TCL1 driving Est2p telomere association throughout the cell cycle via specific interactions with other proteins, Ku80p in G1/early S phase and Est1p in late S/G2 phase.

The G-tail binding Cdc13p is not a telomerase subunit [28]. Therefore, the late S/G2 phase reduction in Est2p telomere binding in cdc13-2 cells [19] (Figure 1B) is unlikely due to a change in telomerase structure. This strain also had low Est1p binding, ~25% of WT levels (Figure 2A) yet Est1p and Est2p abundance

**Figure 4.** Est2p telomere binding in late S/G2 phase is reduced in mutants that lack the stem-bulge region of telomerase RNA. Methods are the same as in Figure 1 legend. A, Est2p binding to VII-L telomere in synchronous tlc1-SD (black diamonds) versus WT (white squares), or untagged (white triangles) cells. Est2p binding to the VII-L telomere was significantly lower in tlc1-SD than in WT cells late in the cell cycle (from 45 to 90 min, P values ranged from P = 0.0007 at 60 min to 0.018 at 90 min). B, Est2p binding to VII-L (top) or VI-R (bottom) telomeres in asynchronous WT and mutant cells. Bar graphs show average Est2p association with telomere VII-L (dark grey) or VI-R (light grey) with error bars indicating ± one standard deviation from that average; abbreviations for strains are SD, SC, CD, and BD, tlc1-BD. The level of Est2p telomere binding in tlc1-SD cells was significantly lower than in WT (P = 0.004, VII-L; 0.0003, VI-R) as well as at the VII-L telomere in tlc1-BD cells (P = 0.001). The level of Est2p telomere binding in WT and tlc1-BD cells was not significantly different (P = 0.083, VII-L; 0.573, VI-R). C, Western analysis of Est2p-G8-Myc in extracts from WT and mutant strains. Abbreviations for strains are same as in panel B.

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was normal in cdc13-2 cells (Figure 1C, 2B). These data can be explained if the holoenzyme comes to the telomere (or is held at the telomere) in late S/G2 phase via a specific interaction between Est1p and Cdc13p that is impaired in cdc13-2 cells [17]. Low but detectable Est1p binding in cdc13-2 cells is consistent with the effects of this mutation on telomerase recruitment to a double strand break (DSB) that is generated next to a tract of telomeric DNA. In cdc13-2 cells, Est1p binding to the break was much lower than in WT cells for up to 2 hrs after DSB formation, but at 3 hrs, Est1p binding was 50% of the WT level [29]. Est1p telomere binding was also reduced when it was unable to interact with TLC1 RNA as in tlc1-SD and tlc1-BD cells (Figure 3A, B). The Cdc13p and TLC1 pathways for Est1p recruitment are not redundant as Est1p binding was low when either interaction was disrupted. However, the pathways must be somewhat independent as Est1p levels at the telomere in late S/G2 phase were 25% of WT in both cdc13-2 (Figure 2A) and tlc1-SD cells (Figure 3A), higher than the background level of Est1p telomere binding in est2A (Figure 2C) and tlc1A48 cdc13-2 cells (Figure 6). Likewise, in asynchronous cells, Est1p telomere binding was statistically indistinguishable in tlc1A, tlc1-SD, and tlc1-BD cells. However, in each strain, binding was higher in the mutant than in the no-tag control (Figure 3B). Thus, a small but significant amount of Est1p can associate with telomeres in the complete absence of TLC1 RNA. We speculate that this low level association is due to the Est1p-Cdc13p interaction.

Together with earlier findings, the data presented here support several conclusions. First, the G1 and the late S/G2 phase Est2p recruitment pathways must be the only ones that bring telomerase to yeast telomeres in otherwise WT cells since there was no telomere associated Est2p or Est1p in doubly mutant strains (tlc1\textsuperscript{D}48 cdc13-2, tlc1\textsuperscript{D}48 est1\textsuperscript{D}, yku80-135i tlc1-SD, yku80-135i tlc1-BD; Figure 5, 6). Second, G1 bound Est2p was neither sufficient (e.g., est1\textsuperscript{D}) nor necessary (e.g., tlc1\textsuperscript{D}48) to maintain telomeres by telomerase. In fact, it is possible that the G1 recruitment pathway contributes to telomere length solely by protecting ends from degradation [30]. This view is supported by the finding that 60% of the Est2p that is telomere associated in G1 phase is located at least 100 bps from the chromosome end and thus is not in a position to lengthen telomeres [31]. Third, Est1p interacts poorly with Cdc13p unless it is part of the holoenzyme. Three est mutants, tlc1-SD, tlc1-BD (Figure 3C) and est2A (Figure 2B) had
WT levels of Est1p, but there was low (tlc1-SD, tlc1-BD; Figure 3A, B) or no (est2A; Figure 2C) Est1p at the telomere in these strains. Thus, in vivo, the interaction between Cdc13p and Est1p detected by biochemical and genetic methods [15,16] either does not occur or is not stable at telomeres unless Est1p is part of the holoenzyme. Fourth, while reduced Est2p binding in late S/G2 phase correlated with an inability to maintain telomeres by telomerase, it was not sufficient to confer an est phenotype. The levels of telomere associated Est2p and Est1p in the non-senescing tlc1A48, yku80-135i, ykuΔ and tel1Δ strains [31,32] are similar to what was seen here for four est mutants (Figure 1A, B; Figure 4A, B), yet telomeres in tlc1A48, yku80-135i, ykuΔ, and tel1Δ cells, while shorter than WT, are stable [22,23,33].

There are several mutually non-exclusive explanations for why similarly low levels of telomerase in late S/G2 phase support telomerase proficiency in some backgrounds (eg, tel1Δ, tlc1A48, yku80-135i, ykuΔ) and an est phenotype in others (cdc13-2, tlc1-BD, tlc1-SD). For example, there may be fairly subtle quantitative differences between Est2p and/or Est1p binding between the two mutant classes that are not detected by ChIP. Alternatively, there may be qualitative differences between the telomerase that is telomerase associated in the two classes of mutants, such as post-translational modification of telomerase subunits or the presence of Est3p. Another possibility is that telomere structure is different between EST1 and est strains in late S/G2 phase, and the telomeric structure found in est cells makes it harder for low amounts of telomerase to engage properly with chromosome ends. The idea that a specific telomere structure is required for telomerase activity is supported by the observation that forced association of Est1p and Est3p with Est2p in G1 phase cells is not sufficient to support telomerase-mediated telomere elongation [18].

From yeasts to humans, the amount of telomerase per cell is surprisingly low [34,35]. Reducing this already low level by mutation in one of several telomerase components [36–39] or by altering a telomere structural protein [40] can cause fatal diseases, such as dyskeratosis congenita or idiopathic pulmonary fibrosis [41,42]. Here we show that in some genetic backgrounds ~50% of WT levels of Est2p at yeast telomeres in late S/G2 phase is not sufficient to maintain telomeric DNA and prevent cellular senescence. An understanding of why yeast cells are sensitive to reduced levels of telomerase in some genetic backgrounds but not in others may help clarify why even relatively modest reductions in telomerase levels in human stem cells affects their survival.

**Materials and Methods**

**Yeast Strains and Plasmids**

All experiments were carried out in YPH499 [43] background that was modified by insertion of URA3 immediately adjacent to the left telomere of chromosome VII [44] to generate YPH499-UT, and the BAR1 gene was deleted and replaced with kanMX6 [21]. Proteins were epitope tagged at their endogenous loci as described [11,21,25] in a manner that places TRP1 at the tagged locus. Briefly, Est1 was tagged at its carboxyl terminus with nine Myc epitopes [19], and Est2p was tagged at its carboxyl end with a Gly8 linker followed by 18 Myc epitopes [21,25]. Cdc13p was tagged at its carboxyl terminus with 9 Myc epitopes [19]. Complete deletions of TLC1 (replaced by LEU2), EST1 (replaced by HIS3), and EST2 (replaced by HIS3) were generated using PCR-mediated transformation [45]. The TLC1 alleles tlc1-SD (stem disruption; 3 bp disrupted in predicted stem), tlc1-SC (stem disruption compensatory; potential for base-pairing restored to tlc1-SD), and tlc1-BD (bulge deletion; deletion of 5 bulged nucleotides) described in [24] were generously provided by Tom Cech and introduced by integration into the genome. The tlc1A48 and yku80-135i alleles are described in [23] and were generously supplied by Dan Gottschling. The cdc13-2 [16], est1A, est2A, tlc1A48, and yku80-135i mutations were generated as heterozygous diploids expressing Myc-tagged proteins. Doubly mutant strains were derived from heterozygous diploids at both loci that also expressed the desired Myc-tagged protein. In both cases, the heterozygous diploids were sporulated. Freshly dissected spores of the desired genotypes were identified by replica plating, grown up, and used immediately for ChIP analyses.
Two Pathways Recruit Telomerase to Yeast Telomeres

Synchrony and ChIP Methods

The ChIP experiments were carried out, analyzed by multiplex PCR, and quantified exactly as described [21]. Briefly, relative fold enrichment of a protein with telomeres was determined by \((\text{TEL}_{\text{IP}}/\text{ARO}_{\text{IP}})/(\text{TEL}_{\text{input}}/\text{ARO}_{\text{input}})\) where input is the amount of the DNA sequence that was PCR amplified in the samples before precipitation and IP is the amount of the sequence in the anti-Myc immuno-precipitate. Cell synchrony experiments were carried out as in [21]. Briefly, 30°C grown, log phase cells (A660 = 0.5) were arrested in late G1 phase using alpha factor (Sigma), removed from alpha factor (0 minutes), and then allowed to proceed through G1 into a synchronous culture at 24°C. Samples were removed at 15 min intervals and processed for ChIP and FACS (fluorescent activated cell sorting) analysis at each time point. For synchrony experiments, the data for each time point are presented as the mean of the three or more independent synchrony experiments plus or minus one standard deviation from the mean. Likewise, values for asynchronous cultures are the mean plus or minus one standard deviation for three independent cultures. Statistical significance was determined using a two-tailed Student’s t test. For the purposes of this paper, \(P \leq 0.05\) were considered significant.

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Author Contributions

Conceived and designed the experiments: JBB VAZ. Performed the experiments: AC JBB. Analyzed the data: AC JBB VAZ. Wrote the paper: AC JBB VAZ.

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