THE UNIVERSITY of EDINBURGH

Edinburgh Research Explorer

Cdk1-Dependent Phosphorylation of Cdc13 Coordinates Telomere Elongation during Cell-Cycle Progression

OPEN ACCESS

Cdk1-Dependent Phosphorylation of Cdc13 Coordinates Telomere Elongation during Cell-Cycle Progression

Shang Li,[1] Svetlana Makovets,[1] Tetsuya Matsuguchi,[1] Justin D. Blethrow,[2] Kevan M. Shokat,[2] and Elizabeth H. Blackburn[1,*]
[1]Department of Biochemistry and Biophysics, University of California, San Francisco, Box 2200, San Francisco, CA 94143-2200, USA
[2]Department of Cellular and Molecular Pharmacology, University of California, San Francisco, CA 94143-2200, USA
*Correspondence: elizabeth.blackburn@ucsf.edu
DOI 10.1016/j.cell.2008.11.027

SUMMARY

Elongation of telomeres by telomerase replenishes the loss of terminal telomeric DNA repeats during each cell cycle. In budding yeast, Cdc13 plays an essential role in telomere length homeostasis, partly through its interactions with both the telomerase complex and the competing Stn1-Ten1 complex. Previous studies in yeast have shown that telomere elongation by telomerase is cell cycle dependent, but the mechanism underlying this dependence is unclear. In *S. cerevisiae*, a single cyclin-dependent kinase Cdk1 (Cdc28) coordinates the serial events required for the cell division cycle, but no Cdk1 substrate has been identified among telomerase and telomere-associated factors. Here we show that Cdk1-dependent phosphorylation of Cdc13 is essential for efficient recruitment of the yeast telomerase complex to telomeres by favoring the interaction of Cdc13 with Est1 rather than the competing Stn1-Ten1 complex. These results provide a direct mechanistic link between coordination of telomere elongation and cell-cycle progression in vivo.

INTRODUCTION

In most eukaryotes, the ends of linear chromosomes are capped by telomeres. Telomeres are essential for both the stability of linear chromosomes and the complete replication of genomic information (Blackburn, 2000). Telomeres are maintained by telomerase, whose activity is highly regulated. In yeast, more than 150 genes affect telomere length maintenance (Askree et al., 2004; Gatbonton et al., 2006). In humans, haploinsufficiency for telomerase RNA in dyskeratosis congenita patients leads to progressive bone marrow failure and premature aging (Chen and Greider, 2004). Hence even a reduction in gene dosage has severe clinical consequences, highlighting the importance of telomerase regulation.

In the budding yeast *S. cerevisiae*, the telomeric DNA consists of 250–350 base pairs of double-stranded $C_{1-3}A/TG_{1-3}$ repeats with a short single-stranded TG_{1-3} 3′ overhang (Wellinger et al., 1993; Zakian, 1996). Sequence-specific telomeric DNA-binding proteins and their associated factors form a dynamic structure at telomeres (Laroche et al., 2000; Smith et al., 2003). This dynamic nucleoprotein structure is essential for telomere silencing, telomere end protection, and telomere length regulation. In *S. cerevisiae*, two major telomeric DNA-binding proteins are Rap1 and Cdc13. While Rap1 binds duplex $C_{1-3}A/TG_{1-3}$ DNA repeats (Conrad et al., 1990; Wright et al., 1992; Wright and Zakian, 1995), Cdc13 binds the single-stranded TG_{1-3} 3′ overhang (Bourns et al., 1998). Cdc13 has been implicated as the major regulator of telomere access by the telomerase complex (Evans and Lundblad, 1999; Hughes et al., 2000; Lendvay et al., 1996; Lin and Zakian, 1996; Nugent et al., 1996). Cdc13 can interact with two distinct complexes that either positively or negatively regulate telomere elongation (Chandra et al., 2001). Association of Cdc13 with Est1 is essential for recruitment of the telomerase holoenzyme, which contains the protein catalytic subunit Est2 and the integral RNA template TLC1, as well as Est1 and Est3 (Lendvay et al., 1996). Cdc13 also interacts with the Stn1-Ten1 protein complex, which negatively regulates telomere elongation and plays an essential role in telomere end protection (Grandin et al., 2001). Hence, Cdc13 tightly regulates telomere elongation through its interactions with both the Est1-Est2-Est3-TLC1 complex and the competing Stn1-Ten1 complex (Chandra et al., 2001). However, the mechanism by which Cdc13 coordinates the binding of these two complexes during telomere elongation had not previously been analyzed.

Telomere length homeostasis is maintained through a dynamic process. Telomerase extends the telomeric TG_{1-3} single-stranded DNA overhang by copying the intrinsic telomerase RNA template, while lagging strand synthesis by DNA polymerases α and δ is inferred to fill the terminal 5′ gap during each cell cycle (Diede and Gottschling, 1999). Previous studies have shown that telomere elongation by telomerase is restricted to late S to G2 phases in vivo (Diede and Gottschling, 1999; Marcand et al., 2000). The timing of telomere elongation in late S and G2 phases correlates with the binding of protein factors involved in telomere elongation, including Est1, Est2, and Cdc13 (Schramke et al., 2004; Taggart et al., 2002). These data have suggested that the assembly of a functional telomerase complex at the telomeres is restricted to late S to G2 phases of the cell cycle.

In budding yeast, the G-rich overhang is very short (about 13 bases) throughout most of the cell cycle but becomes longer around late S to G2 phases (Larrivee et al., 2004; Wellinger et al., 1993). Available data have suggested that Cdk1 activity is required for the generation of this extended 3′ single-strand overhang, although the details of the mechanism were unknown (Frank et al., 2006; Vodenicharov and Wellinger, 2006). Increased binding of Cdc13 to such an extended 3′ single-strand overhang could serve to subsequently recruit telomerase through the interaction of Cdc13 with Est1. Of the four telomerase components, Est1, Est2, TLC1, and Est3, only the expression of Est1 is cell cycle regulated, peaking at late S and G2 phases (Osterhage et al., 2006). Hence, expression of Est1 at late S and G2 phases likely restricts the assembly of functional telomerase complex to late S and G2 phases. How cells coordinate cell-cycle progression and the recruitment of telomerase complex to telomere has been an open question.

In budding yeast, the regulation of cell-cycle progression depends on a single cyclin-dependent kinase, Cdk1 (Cdc28). Cdk1 regulates cell-cycle progression by phosphorylating hundreds of different protein substrates (Ubersax et al., 2003). The association with various, periodically expressed cyclins regulates the substrate specificity of Cdk1. While it is known that telomere elongation is cell cycle dependent, no Cdk1 substrates that regulate telomere elongation have been identified. Here we show that Cdk1-dependent phosphorylation of Cdc13 at threonine 308 plays an important role in the efficient recruitment of the telomerase complex to telomeres in late S to G2 phases of the cell cycle. Both the telomerase complex and the Stn1-Ten1 complex are recruited to telomeres during late S and G2 phases of cell-cycle progression. Therefore, since these two complexes counteract each other in terms of telomere length regulation, it is necessary to coordinate their binding to telomeres in order to ensure active telomerase function. Our data show that phosphorylation of Cdc13 by Cdk1 plays such a key regulatory role, by coordinating the subsequent recruitment of these two complexes to telomeres to ensure proper telomere elongation and telomere protection.

RESULTS

Cdc13 Is Phosphorylated by Cdk1-as1 In Vitro

To identify potential Cdk1 substrates among components of the telomerase and telomere complexes that might contribute to the coordination of telomere elongation and cell-cycle progression in budding yeast, we utilized a previously developed strategy for specific labeling of substrates by a single kinase (Ubersax et al., 2003). In this approach, the conserved phenylalanine 88 in the ATP-binding domain of Cdk1 is replaced by glycine (Cdk1-as1). Previous data have shown that Cdk1-as1 is functional in vivo and exhibits a much higher affinity and selectivity for the bulky ATP analog N^6-Benzyl-ATP (Bishop et al., 2000; Ubersax et al., 2003). Cdk1-as1/cyclin complexes were purified from an asynchronous yeast culture (Figure 1A). Thus, the purified Cdk1-as1/cyclin complexes contain various Cdk/cyclin complexes with kinase activity for different cell-cycle stages. We chose this strategy because we did not know when any potential Cdk1 substrates from telomerase and telomere complexes might be phosphorylated in vivo. For in vitro kinase

assays using this preparation, 6xHis tagged recombinant protein versions of two telomerase subunits (Est1 and Est3) and two telomere-binding factors (Cdc13 and Ten1) were used as substrates (Figure 1B). As a control for Cdk1-as1 phosphorylation specificity, we used a 6xHis-Cdc13-7A, in which alanine residues replace all seven predicted Cdk1 phosphorylation sites (as indicated in Figure S1A available online). Figure 1C shows that only wild-type Cdc13 recombinant protein is specifically phosphorylated by the Cdk1-as1 in the presence of $[\gamma^{-32}P]N^6$-Benzyl-ATP, with only background phosphorylation detected for 6xHis-Cdc13-7A, 6xHis-Est1, 6xHis-Est3, and 6xHis-Ten1. For Est2 and Stn1, insufficient protein was obtained from the bacterial expression system. Instead, we used partially purified Est2-13myc and Stn1-13myc proteins from yeast lysates as substrates. However, no specific Cdk1-as1 phosphorylation was identified for these proteins in vitro (data not shown).

To identify the putative Cdk1 phosphorylation sites in Cdc13 phosphorylated by Cdk1-as1 in vitro, we engineered and purified 6xHis-Cdc13 recombinant proteins containing an alanine mutation at each of the seven individual potential Cdk1 phosphorylation sites in turn (as indicated in Figure S1A). As shown in Figure 1D, mutation of Cdc13 threonine 308 to alanine (T308A) dramatically reduced the phosphorylation by Cdk1-as1 in vitro. Since some residual phosphorylation of Cdc13 was seen in the presence of the single threonine 308 to alanine mutation (Figure 1D, bottom panel, compare lanes 4 and 6), we further engineered and purified 6xHis-Cdc13 recombinant proteins containing the T308A mutation in combination with each of the other six additional potential Cdk1 phosphorylation site mutations. As shown in Figure 1E, when both threonine 308 and serine 336 in Cdc13 were mutated to alanine, no phosphorylation above background was observed (Figure 1E, bottom panel, compare lanes 3 and 6). Mutation of the other five putative Cdk1 phosphorylation sites in addition to the T308A mutation did not affect phosphorylation by Cdk1-as1 in vitro. These results suggested that both threonine 308 and serine 336 could be phosphorylated by Cdk1-as1 in vitro.

Phosphorylation of Cdc13 by Cdk1 In Vivo

To determine whether both Cdc13 threonine 308 and serine 336 are phosphorylated in vivo, we first asked whether mutation of either threonine 308 or serine 336 affected telomere length regulation. A diploid yeast strain (A364a background) heterozygous for the threonine 308 to alanine mutation cdc13-T308A or the serine 336 to alanine mutation cdc13-S336A was sporulated and dissected. The telomere length of each individual yeast colony derived from the dissected spores was measured after ~100 population doubling (PD100). Southern blot hybridization using a telomere probe (teloblot) showed that the cdc13-S336A mutation did not affect telomere length maintenance (Figure 2, compare lanes 1 and 2 to lanes 3 and 4). In contrast, the cdc13-T308A mutation resulted in 75 ± 10 bp telomere shortening (a 25% reduction in normal telomere length; Figure 2, compare lanes 5 and 6 to lanes 7 and 8). Such telomere shortening had largely taken place by ~50 cell divisions after sporulation (Figure S2). Similar results were observed in the S288C yeast strain background (Figure S3A). Simultaneous mutation of threonine 308 and serine 336 to alanine or mutation of all seven
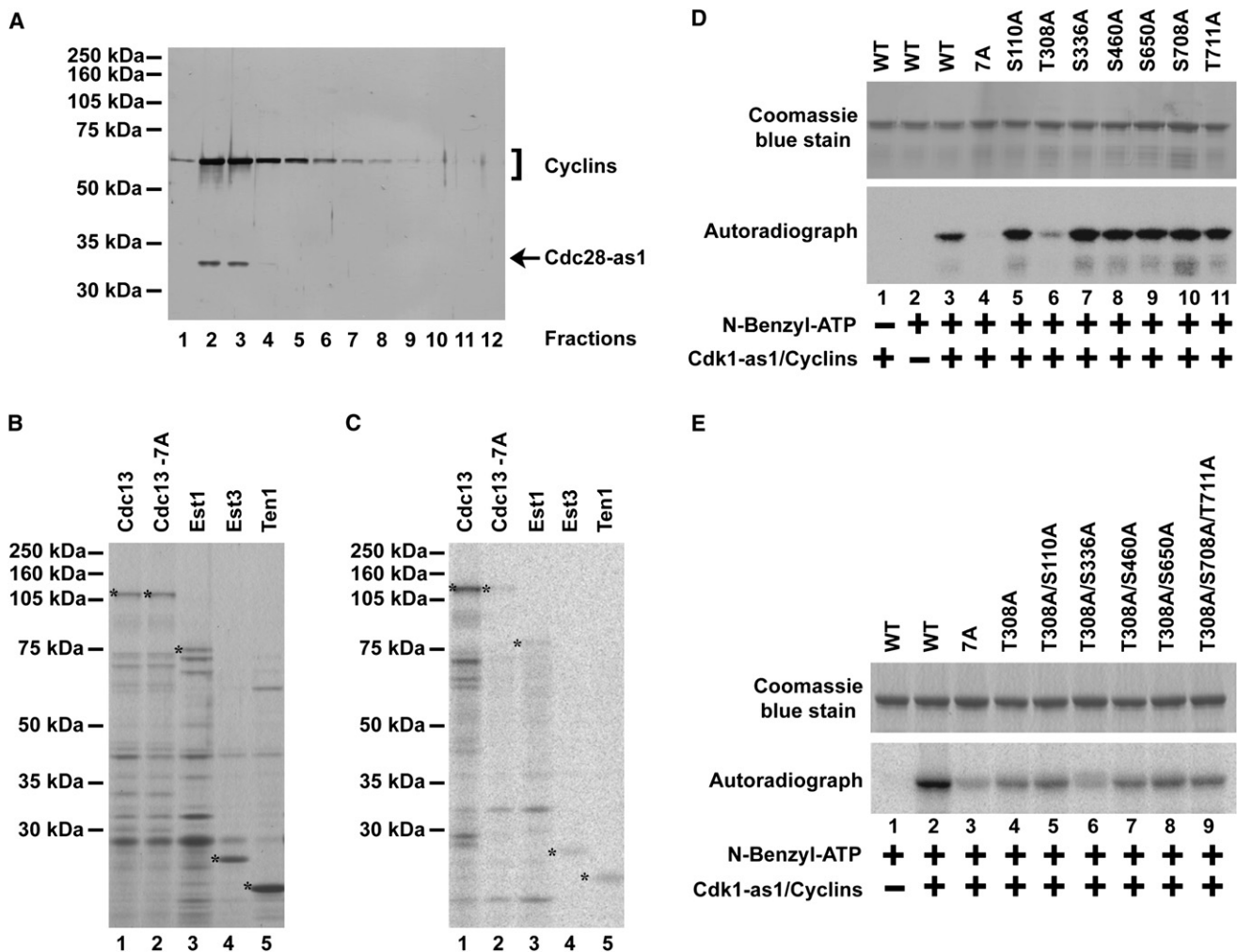
Figure 1. Phosphorylation of Cdc13 by Cdk1-as1 In Vitro

(A) A silver-stained gel showing the purification of TAP-tagged Cdk1-as1 and copurified cyclins from asynchronous yeast cell cultures.

(B) Coomassie blue stain gel showing the input of recombinant 6xHis tagged telomerase subunits and telomere-binding proteins. Cdc13-7A is used as negative control with all seven potential Cdk1 phosphorylation sites mutated to alanine. Asterisks mark proteins of expected molecular weight.

(C) Autoradiograph showing the specific phosphorylation of wild-type Cdc13 by Cdk1-as1/cyclin complexes in vitro in the presence of $[\gamma\text{-}^{32}P]N^6$-Benzyl ATP but not Cdc13-7A, Est1, Est3, or Ten1.

(D) In vitro phosphorylation assay of mutant Cdc13 proteins that contain mutations at individual putative Cdk1 phosphorylation site as indicated. Mutant Cdc13 with threonine 308 to alanine mutation results in dramatic reduction of phosphorylation by Cdk1-as1/cyclins in vitro.

(E) In vitro phosphorylation assay of mutant Cdc13 proteins that contain putative Cdk1 phosphorylation site mutations in addition to threonine 308 to alanine mutation. Mutation of both threonine at position 308 and serine at position 336 results in complete loss of phosphorylation by Cdk1-as1 in vitro.

putative Cdk1 phosphorylation sites to alanine caused telomere shortening similar to that of *cdc13-T308A* alone (Figure 2, compare lanes 9 and 10 to lanes 11 and12; lanes 13 and 14 to lanes 15 and 16). Replacement of Cdc13 threonine 308 with aspartic or glutamic acid to provide a negative charge similar to phosphorylation failed to rescue the telomere shortening phenotype (Figure 2, compare lanes 17 and 18 to 19 and 20; lanes 21 and 22 to 23 and 24), suggesting that phosphorylation itself, rather than the negative charge associated with the phosphorylation event, is important for Cdc13 function in vivo.

Since mutation of Cdc13 threonine 308 to alanine results in dramatic reduction of phosphorylation by Cdk1-as1 in vitro

and significant telomere shortening in vivo, these findings suggested that Cdc13 threonine 308 may be phosphorylated and this phosphorylation event may be necessary for normal telomere maintenance in vivo. Consistent with this hypothesis, analysis of the Cdc13 amino acid sequence alignments across different fungal species in the yeast genome database shows that Cdc13 threonine 308 is well conserved, but serine 336 is not (Figure S1B).

Mutation of threonine 308 to alanine did not result in obvious changes in Cdc13 protein mobility on SDS-PAGE (data not shown). To ask whether Cdc13 threonine 308 is phosphorylated in vivo, we raised a Cdc13 threonine 308 phospho-specific rabbit
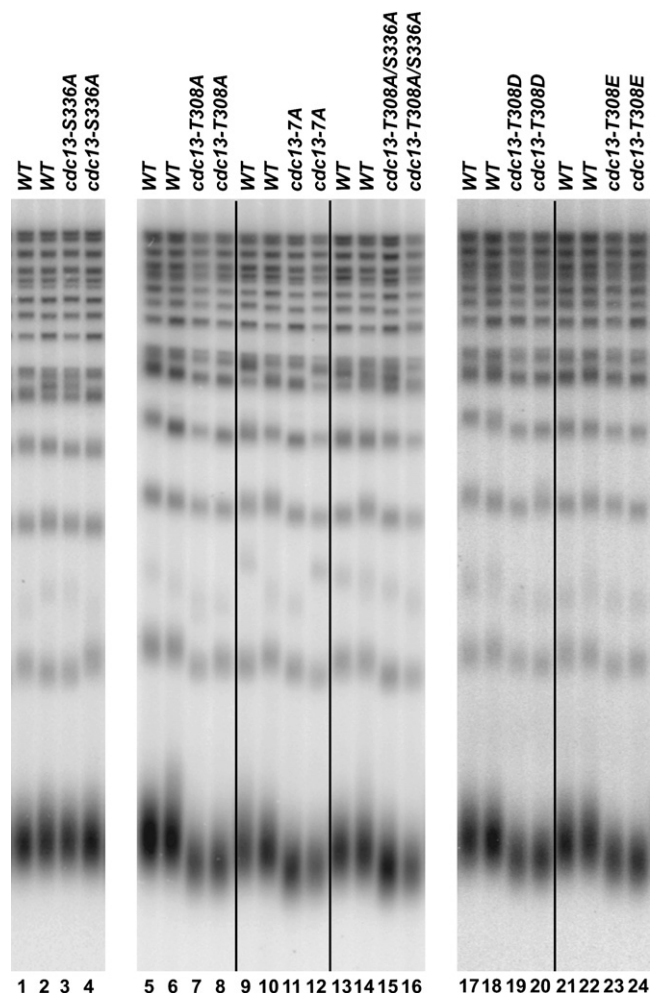
Figure 2. Cdc13 Threonine 308 Mutation Results in Telomere Shortening In Vivo

Diploid yeast strains heterozygous for wild-type and putative Cdk1 phosphorylation site mutations in *CDC13* were sporulated and dissected. The telomere length of each individual yeast colony derived from the dissected spore was measured at ~PD100. Mutation of *CDC13* threonine 308 to alanine results in telomere shortening (lanes 5–8).

polyclonal antibody. As shown in Figure 3A, this Cdc13 threonine 308 phospho-specific antibody recognized endogenously expressed wild-type Cdc13-13myc protein immunoprecipitated from asynchronous A364a yeast cell lysate but not the Cdc13-T308A-13myc mutant (Figure 3A, top panel). Reprobing the same western blot with anti-myc antibody showed that the expression levels of Cdc13-13myc and Cdc13-T308A-13myc mutant proteins are comparable (Figure 3A, bottom panel), suggesting that this threonine 308 phospho-specific antibody is specific. As shown in Figure 3B, lambda phosphatase treatment results in the loss of Cdc13 detection by the phospho-specific antibody, while untreated lysate or lysate treated with lambda phosphatase in the presence of phosphatase inhibitor still results in specific detection of Cdc13 by the threonine 308 phospho-specific antibody (Figure 3B, top panel, compare lane 3 to lanes 1 and 2). These data confirm that Cdc13 threonine 308 is phosphorylated in vivo.

To confirm that Cdc13 threonine 308 is phosphorylated by Cdk1, it is important to show that the phosphorylation of threonine 308 in vivo is dependent on Cdk1 activity. Previous studies have shown that in a yeast strain carrying the Cdk1-as1 allele, the activity of CDK can be inhibited by 5–25 μM of 1-NM-PP1 within 10 min (Bishop et al., 2000). This rapid inhibition of Cdk1-as1 activity by 1-NM-PP1 reduces the potential for indirect effects due to shift of cell-cycle position. As shown in Figure 3C, addition of 10 μM 1-NM-PP1 to asynchronous yeast culture at 30°C results in rapid loss of Cdc13 detection by the threonine 308 phospho-specific antibody within 15 min (Figure 3C, top panel, compare lane 1 to lanes 2 and 3). These data suggest that Cdc13 threonine 308 is phosphorylated by Cdk1 in vivo. Treatment of the asynchronous yeast culture with 1-NM-PP1 also results in some enrichment of yeast in G1 phase of the cell cycle, as shown by FACS analysis of cellular DNA content (Figure 3D, compare 0 min to 15 and 30 min time points).

To identify the specific cell-cycle stages during which Cdc13 is phosphorylated, alpha-factor synchronized yeast cultures were analyzed in a series of time points every 15 min. As shown in Figure 3E, phosphorylation of Cdc13 can be readily detected in the time span from 45 min to 75 min after alpha-factor release. FACS analysis of cellular DNA content suggested that the phosphorylation of Cdc13 occurred in late S to G2 phases of the cell cycle (Figure 3F), which coincides with the ability of telomerase to perform telomere elongation in vivo, as shown previously (Diede and Gottschling, 1999; Marcand et al., 2000; Schramke et al., 2004; Taggart et al., 2002). Consistent with these data obtained in the A364a yeast strain background, phosphorylated Cdc13 could also be detected in asynchronous S288C yeast lysates but not from alpha-factor arrested S288C cell lysate (Figure S3B).

Cdc13 Phosphorylation Affects Telomerase-Dependent Telomere Maintenance

To further understand the functional significance of Cdk1-dependent phosphorylation of Cdc13, we tested whether the phosphorylation of Cdc13 threonine 308 is involved in telomerase-dependent or telomerase-independent telomere maintenance pathways. For this purpose, we compared the telomere length in the *cdc13-T308A* single mutant with single mutants of known telomerase-dependent telomere maintenance factors (*est2Δ*, *tlc1Δ*, *tel1Δ*, *mec1Δ*, *yku70Δ*, and *yku80Δ*) as well as in double mutants containing *cdc13-T308A* in combination with *est2Δ*, *tlc1Δ*, *tel1Δ*, *mec1Δ*, *yku70Δ*, or *yku80Δ*. As shown in Figure S4, mutation of Cdc13 threonine 308 to alanine did not result in additive or synergistic telomere shortening phenotypes with *est2Δ*, *tlc1Δ*, *tel1Δ*, *mec1Δ*, *yku70Δ*, or *yku80Δ* deletions, indicating that Cdk1-dependent phosphorylation of Cdc13 likely plays a role in the telomerase-dependent telomere maintenance mechanism. As predicted from these findings, no accelerated senescence phenotype was observed in *cdc13-T308A est2Δ* or *cdc13-T308A tlc1Δ* double mutants compared to *est2Δ* or *tlc1Δ* single mutants (Figure S5).

Cdc13 Phosphorylation Is Necessary for Efficient Telomerase Recruitment to the Telomeres

Several functional domains have been identified in Cdc13, as shown in Figure S6A. Previous data from yeast two-hybrid
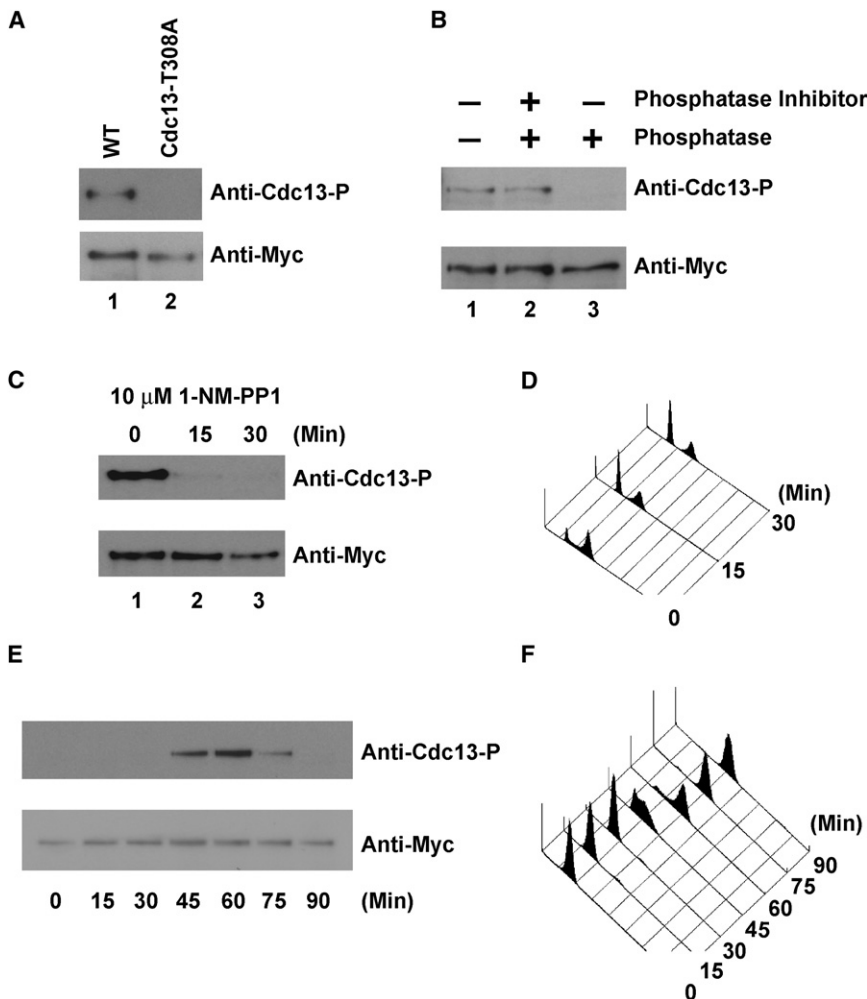
Figure 3. Cdk1-Dependent Phosphorylation of Cdc13 Threonine 308 during Late S/G2 Phases of Cell Cycle

Endogenously expressed Cdc13 is tagged at its C terminus with a 13-myc tag.

(A) Cdc13 phospho-threonine 308-specific antibody detected wild-type Cdc13 but not Cdc13-T308A mutant protein from asynchronous yeast cell lysates (top panel). The same blot was stripped, then reprobed with anti-myc antibody to detect the total amount of Cdc13 protein (bottom panel).

(B) Threonine 308 phosphorylation is sensitive to lambda phosphatase treatment.

(C) Inactivation of Cdk1-as1 activity by 1-NM-PP1 results in rapid loss of Cdc13 phosphorylation in vivo. An asynchronous yeast culture was treated with 10 μM 1-NM-PP1 and harvested 0, 15, or 30 min after treatment.

(D) FACS analysis shows slight changes of cell-cycle progression after 1-NM-PP1 treatment.

(E) Phosphorylation of Cdc13 threonine 308 is cell cycle dependent, enriched around 45–75 min after alpha-factor synchronization release.

(F) FACS analysis showing cell-cycle progression after alpha-factor synchronization. At 45–75 min after alpha-factor synchronization release, the cell cycle has progressed into late S to G2 phases.

analysis also identified several protein interaction regions in Cdc13, including the N-terminal region (amino acids 1–252) that is necessary for interaction with Pol1, Imp4, Sir4, and Zds2 (Hsu et al., 2004), the Est1-interacting region (recruitment domain, RD) from amino acids 190–340, and the Stn1-interacting region from amino acids 252–924 (Figure S6A) (Petreaca et al., 2006). To understand the molecular function of Cdc13 phosphorylation, we first tested whether the mutation of Cdc13 threonine 308 to alanine affects the recruitment of Cdc13 to telomeres during cell-cycle progression. Chromatin immunoprecipitation (ChIP) and Q-PCR analysis (Figure 4A) showed that wild-type Cdc13 is recruited to telomeres in a cell-cycle-dependent manner, as previously reported (Schramke et al., 2004; Taggart et al., 2002). Mutation of threonine 308 to alanine did not affect this cell-cycle-dependent recruitment of Cdc13 to telomeres (Figures 4A and S7). These results are consistent with the location of threonine 308 being outside the Cdc13 DNA-binding motif (Figure S6A). Mutation of threonine 308 to alanine also did not significantly affect the cell-cycle-regulated generation of single-stranded G-rich overhangs (Figure S8). Although the single-stranded overhang signal in *CDC13* yeast appears slightly stronger than that in *cdc13-T308A* yeast during S/G2, this is

likely due to the fact that the *CDC13* yeast is more efficient in elongating telomeres (during S/G2 phase) and hence elongating the G-rich strand more.

Since threonine 308 lies in both the Est1- and the Stn1-interacting regions (Figure S6A), we next asked whether Cdk1-dependent phosphorylation of Cdc13 plays a role in coordinating the interaction of Cdc13 with Est1 and Stn1. As previously reported, we could not detect the interaction of endogenous Cdc13 with Est1 or Stn1 by coimmunoprecipitation (Chandra et al., 2001; Petreaca et al., 2007). However, when 13myc-Est1 or 13myc-Stn1 was overexpressed using a galactose-inducible promoter, we readily detected the interaction of 13myc-Est1 or 13myc-Stn1 with endogenously expressed Cdc13-3HA. Mutation of threonine 308 to alanine did not abolish the interaction of Cdc13 with Est1 or Stn1 (Figure 4B, top panel, compare lanes 3 and 4, lanes 5 and 6), although the interaction of Cdc13-3HA with 13myc-Est1 seems to be slightly stronger than that of Cdc13-T308A-3HA (Figure 4B, compare lanes 3 and 4 on top panel). To address whether T308A mutation affects the interaction of Cdc13 and Est1 in a quantitative way under physiological conditions, we took advantage of the fact that the interaction of Cdc13 and Est1 is necessary for the telomeric recruitment of telomerase holoenzyme (which contains the integral RNA template component, TLC1) by Cdc13. By using QRT-PCR to analyze the amount of TLC1 that coimmunoprecipitated with Cdc13, we were able to address whether phosphorylation of Cdc13 affects its interaction with Est1 quantitatively. Indeed, mutation of threonine 308 to alanine
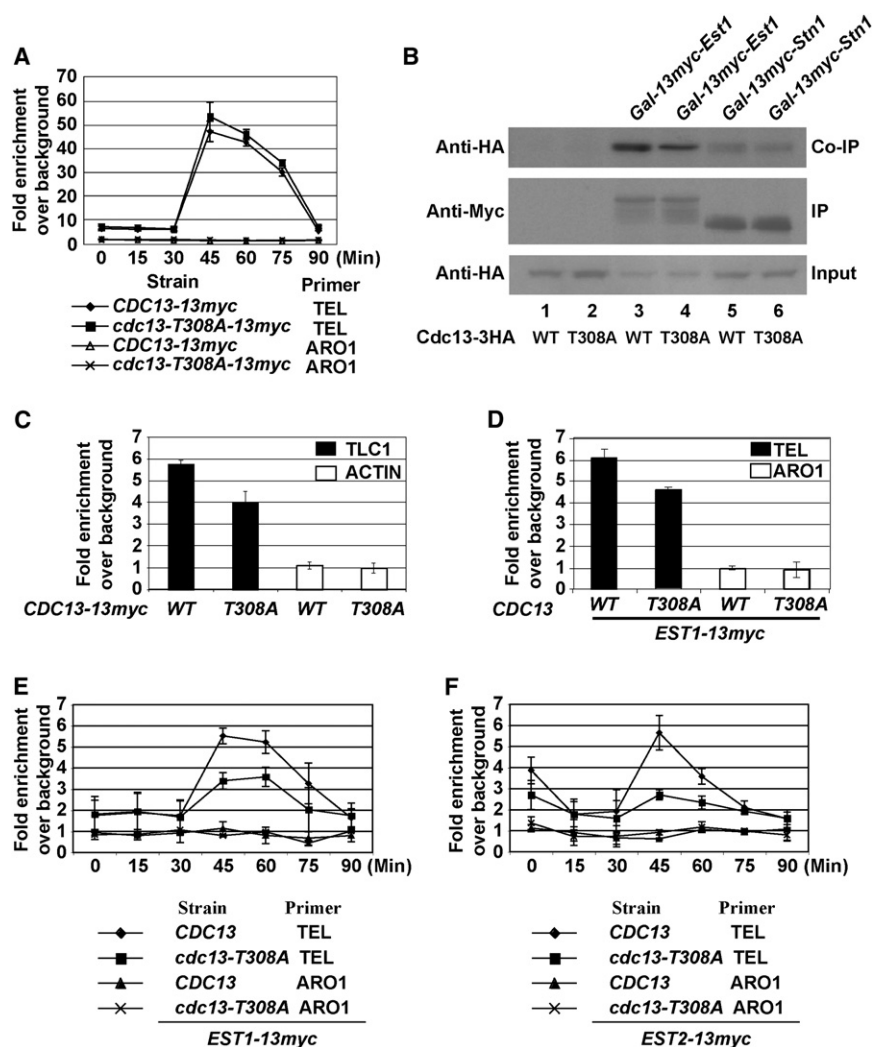
when an individual telomere is shortened—results in telomeres being elongated. Such telomere elongation induced by *rap1ΔC*, *rif1Δ*, or *rif2Δ* is telomerase dependent (Marcand et al., 1997). To ask whether Cdk1-dependent phosphorylation of Cdc13 is important for coordination of this increased telomerase action during cell-cycle progression in yeast lacking Rif1 or Rif2, we first compared the telomere length of haploid strains obtained via dissection of sporulation products from *cdc13-T308A/rif1Δ* or *cdc13-T308A/rif2Δ* diploid heterozygotes. As shown in Figure 5A, the telomere overelongation induced by the absence of Rif1 or Rif2 depended on Cdk1-dependent phosphorylation of Cdc13. This impairment in telomere overelongation in *cdc13-T308A rif1Δ* or *cdc13-T308A rif2Δ* yeast was attributable to lower telomerase recruitment by the mutant Cdc13-T308A compared to wild-type Cdc13, as indicated by reduced association of Cdc13-T308A with TLC1 in coimmunoprecipitation experiments (Figures 5B and S9A). Consistent with the long telomere length in *rif1Δ* or *rif2Δ* cells harboring wild-type Cdc13, a strong increase of telomerase recruitment by Cdc13 was observed, as indicated by increased coimmunoprecipitation of Cdc13 and TLC1. The increase in Cdc13 association with TLC1 is roughly proportional to the telomere overelongation in the strains harboring *rif1Δ* or *rif2Δ* (Figures 5B and S9A). The expression level of endogenous Cdc13 or Cdc13-T308A was not affected by loss of Rif1 or Rif2 (Figure S9B). Since chromatin shearing in the preparation for ChIP using sonication will result in preferential underrepresentation of subtelomeric sequence in yeast with the dramatic telomere lengthening caused by *rif1Δ*, it was difficult to accurately quantify telomere sequence

results in 30% reduction of TLC1 coimmunoprecipitated with Cdc13 but not of the control actin mRNA (Figures 4C and S9A). The recruitment of Est1 to telomeres was also correspondingly reduced by at least 25% in the *cdc13-T308A* mutant as shown by ChIP from unsynchronized yeast culture (Figure 4D). We further demonstrated that cell-cycle-dependent recruitment of Est1 and Est2 to telomeres is also reduced in yeast harboring the Cdc13 threonine 308 to alanine mutation compared to wild-type (Figures 4E, 4F, and S10). Together, these data indicated that Cdk1-dependent phosphorylation of Cdc13 is necessary for maximal recruitment of the telomerase complex to telomeres during cell-cycle progression but is not absolutely required.

In *S. cerevisiae*, the double-stranded telomeric DNA-binding protein Rap1 associates with Rif1 and Rif2 through its C-terminal domain and has been shown to negatively regulate telomere lengthening through a potential Rap1/Rif1/Rif2 counting mechanism (Levy and Blackburn, 2004; Marcand et al., 1997). In yeast strains lacking the Rap1 C terminus, Rif1, or Rif2, telomere length homeostasis is disabled and long telomeres ensue (Levy and Blackburn, 2004; Marcand et al., 1997). Reduction of Rap1, Rif1, and Rif2 association with telomeres—similar to the situation
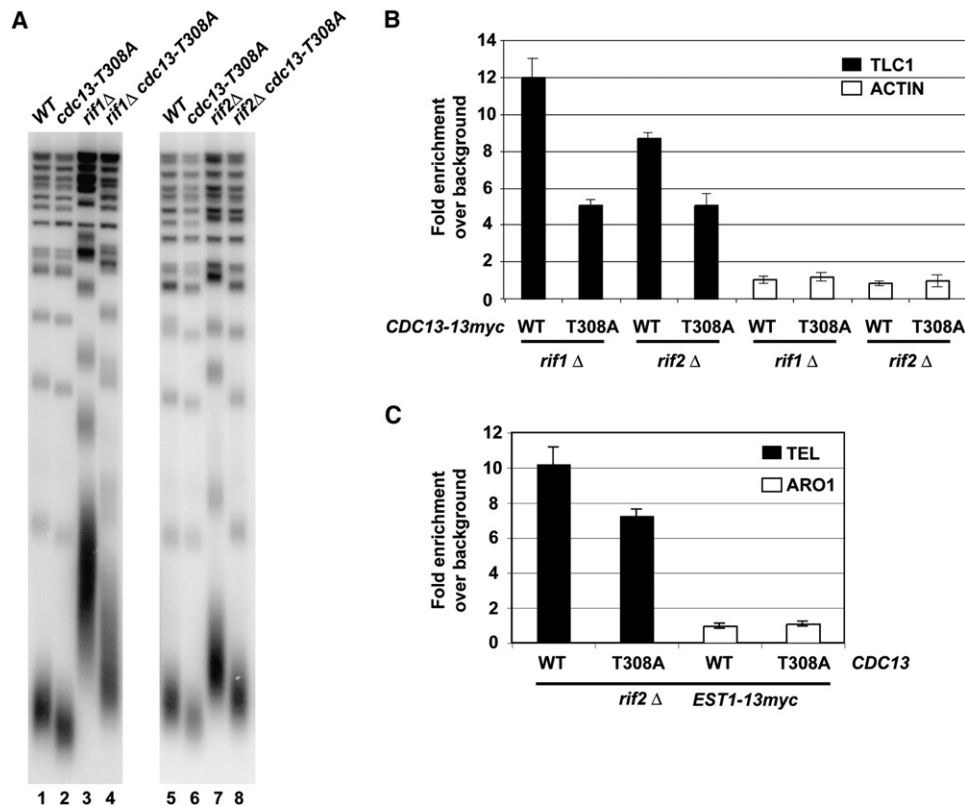
Figure 5. Phosphorylation of Cdc13 Threonine 308 Is Necessary for Efficient Recruitment of the Telomerase Complex to Telomeres in *rif1Δ* or *rif2Δ* Yeast

(A) Rapid telomere elongation in yeast strains with *rif1Δ* or *rif2Δ* is strongly inhibited by Cdc13 threonine 308 to alanine mutation, as shown by teloblot.

(B) Coimmunoprecipitation of Cdc13 and TLC1 in *rif1Δ* or *rif2Δ* yeast. Deletion of *RIF1* or *RIF2* results in a dramatic increase of telomerase recruitment by Cdc13 (as indicated by dramatically increased coimmunoprecipitation of Cdc13 and TLC1 RNA) that is hampered by Cdc13 threonine 308 to alanine mutation (p values < 0.01).

(C) ChIP assays show that recruitment of Est1 to telomeres is compromised in *cdc13-T308A rif2Δ* yeast compared to *CDC13 rif2Δ* yeast expressing Est1-13myc (p values < 0.01).

enrichment using a standard Y′ subtelomeric PCR primer set (data not shown). However, ChIP clearly showed increased Est1 recruitment to telomeres in *CDC13 rif2Δ* yeast, which was reduced in *cdc13-T308A rif2Δ* yeast (Figure 5C). Together these data suggested that phosphorylation of Cdc13 threonine 308 plays a central role in coordinating telomerase recruitment to telomeres during telomere elongation in yeast lacking Rif1 or Rif2.

Stn1-ΔC199 Rescues Cdc13-T308A Deficiency

Since Cdc13 threonine 308 is also located inside the Stn1 interaction domain (Figure S6A), we tested whether Cdc13 phosphorylation affects the recruitment of Stn1 to telomeres. ChIP analysis showed that cell-cycle-dependent recruitment of Stn1-13myc to telomeres (Figures 6A and S7) was generally similar in *CDC13* or *cdc13-T308A* cells. The Stn1 telomere association peaked in late S to G2 phases, coincident with the recruitment of the telomerase complex during cell-cycle progression, as shown previously (Schramke et al., 2004; Taggart et al., 2002). Because both the telomerase complexes and the Stn1-Ten1 complexes are recruited to telomeres during late S and

G2 phases of cell-cycle progression and potentially counteract each other in terms of telomere length regulation, we asked whether the phosphorylation of Cdc13 plays a role in coordinating its interaction with these two distinct complexes. Yeast lacking wild-type Stn1 and expressing Stn1-ΔC199 (which has the C terminus necessary for its interaction with Cdc13 deleted, Figure S6B) are viable but show dramatic telomere lengthening (Petreaca et al., 2006), as shown in Figure 6B. Deletion of *RAD52*, which is required for homologous DNA recombination events in yeast, did not affect telomere elongation in this *stn1-ΔC199* strain, indicating that telomere overelongation in *stn1-ΔC199* strains is due to an effect on telomerase (Figure 6B, compare lanes 1 and 2 to lanes 5 and 6). Therefore, we tested whether the mutation of Cdc13 threonine 308 to alanine affected telomere elongation in *stn1-ΔC199* yeast. In striking contrast to *rif1Δ* or *rif2Δ* yeast, loss of Cdc13 phosphorylation did not affect telomere lengthening in *stn1-ΔC199* yeast (Figure 6B, compare lanes 5 and 6). Consistent with this observation, *stn1-ΔC199* also rescued the inhibition of telomerase recruitment by the *cdc13-T308A* mutation, as indicated by dramatically increased and similar association of Cdc13 and
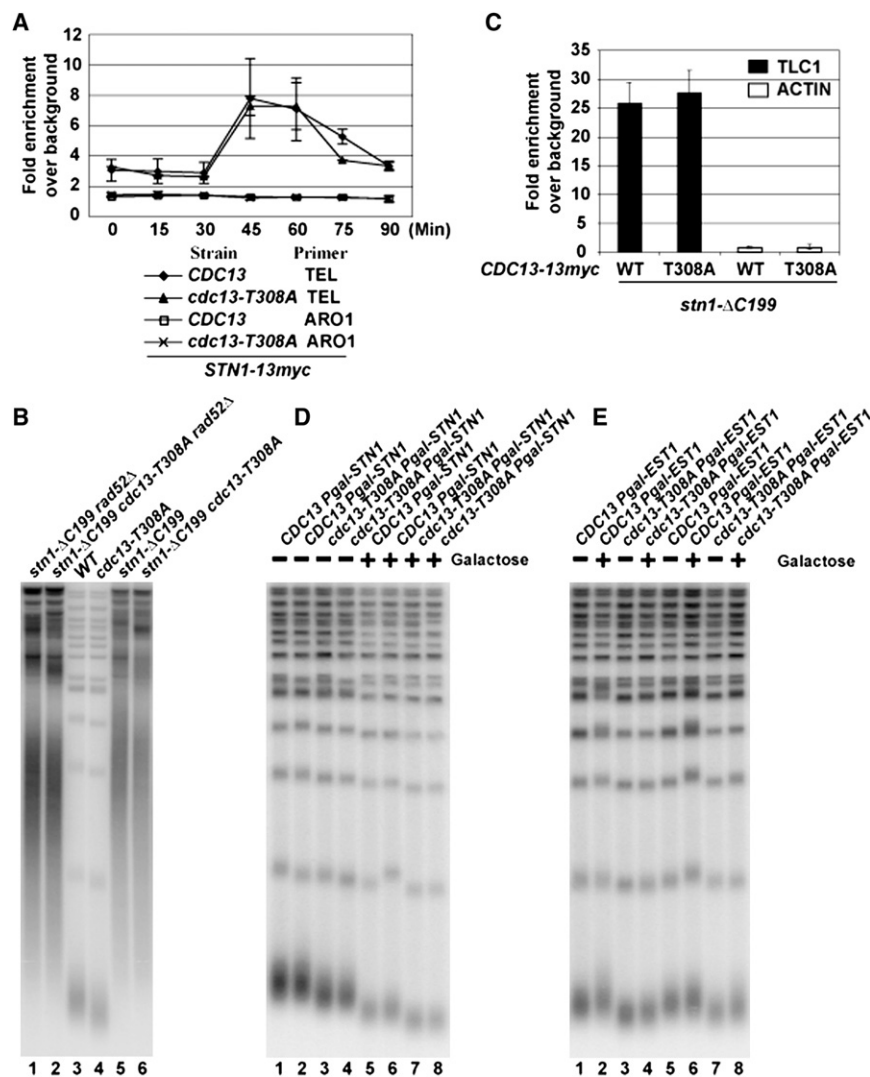
Figure 6. *Stn1-ΔC199* Rescues the Telomere Shortening Phenotype Induced by Cdc13 Threonine 308 to Alanine Mutation

(A) ChIP assays show that recruitment of Stn1 to telomeres is cell cycle regulated in yeast strain expressing Stn1-13myc.

(B) Dramatic telomere lengthening in yeast strain harboring *stn1-ΔC199* mutation is not affected by Cdc13 threonine 308 to alanine mutation (compare lanes 5 and 6) or *RAD52* deletion (compare lanes 1 and 2 to lanes 5 and 6).

(C) Coimmunoprecipitation of Cdc13 and TLC1 in *stn1-ΔC199* yeast. Dramatic increase of telomerase recruitment by Cdc13 in *stn1-ΔC199* yeast is not affected by Cdc13 threonine 308 to alanine mutation (indicated by substantially increased coimmunoprecipitation of both Cdc13 and Cdc13-T308A with TLC1, p values = 0.559).

(D) Overexpression of Stn1 in yeast with wild-type *CDC13* results in telomere shortening. Overexpression of Stn1 in yeast with *cdc13-T308A* results in further telomere shortening.

(E) Overexpression of Est1 in yeast with wild-type *CDC13* results in slight telomere elongation. Overexpression of Est1 in yeast with *cdc13-T308A* also results in slight telomere elongation but not complete rescue of the telomere shortening resulting from the *cdc13-T308A* mutation.

Cdc13-T308A with TLC1 (Figures 6C and S9A). The increased associations of Cdc13 and Cdc13-T308A with TLC1 are also commensurate with the degree of telomere elongation in *stn1-ΔC199* yeast, compared to wild-type yeast (Figure S9A). The expression level of endogenous Cdc13 or Cdc13-T308A was not affected by Stn1-ΔC199 (Figure S9B). Hence, these data provide evidence that interaction of Stn1 and Cdc13 is responsible for the inhibition of telomerase recruitment to telomeres that we observed in *cdc13-T308A* yeast. This is likely due to the increased competition of Stn1-Ten1 complex with telomerase complex for binding to Cdc13 in the absence of Cdk1-dependent phosphorylation. Since it is very difficult to accurately quantify the recruitment of Est1 or Est2 to telomeres using ChIP in *stn1-ΔC199* yeast, due to the extremely long telomeres, it was still possible that the Cdc13-T308A mutation may affect the binding of Stn1 or Est1 to Cdc13 indirectly. To further address these possibilities, we showed that overexpression of Stn1 in yeast with wild-type *CDC13* resulted in telomere shortening (Figure 6D, lanes 5 and 6) as previously shown (Chandra et al., 2001; Dahlseid et al., 2003). Overexpression of Stn1 in

cdc13-T308A yeast resulted in further telomere shortening (Figure 6D, lanes 7 and 8). In contrast, overexpression of Est1 in yeast with wild-type *CDC13* resulted in slight telomere elongation (Figure 6E, lanes 2 and 6). Overexpression of Est1 in yeast with *cdc13-T308A* also resulted in slight telomere elongation but not complete rescue of the telomere shortening attributable to the *cdc13-T308A* mutation (Figure 6E, lanes 4 and 8). In summary, these data indicate that phosphorylation of Cdc13 by Cdk1 plays a central role in regulating the access of the telomerase complex to telomeres via competition with the Stn1-Ten1 complex during cell-cycle progression.

DISCUSSION

We have shown that Cdc13 is phosphorylated by Cdk1 in vivo during late S to G2 phases of the cell cycle, a time in the cell cycle that coincides with the previously reported recruitment of the telomerase complex to telomeres (Schramke et al., 2004; Taggart et al., 2002). Our data provide evidence that phosphorylation of Cdc13 is important in regulating the recruitment efficiency of the telomerase complex to telomeres by competing with the Stn1-Ten1 complexes during telomere elongation. Uncoupling of this cell-cycle-dependent event by inactivation of Cdc13 phosphorylation results in less telomerase recruitment to telomeres during cell-cycle progression and compromised telomere elongation.
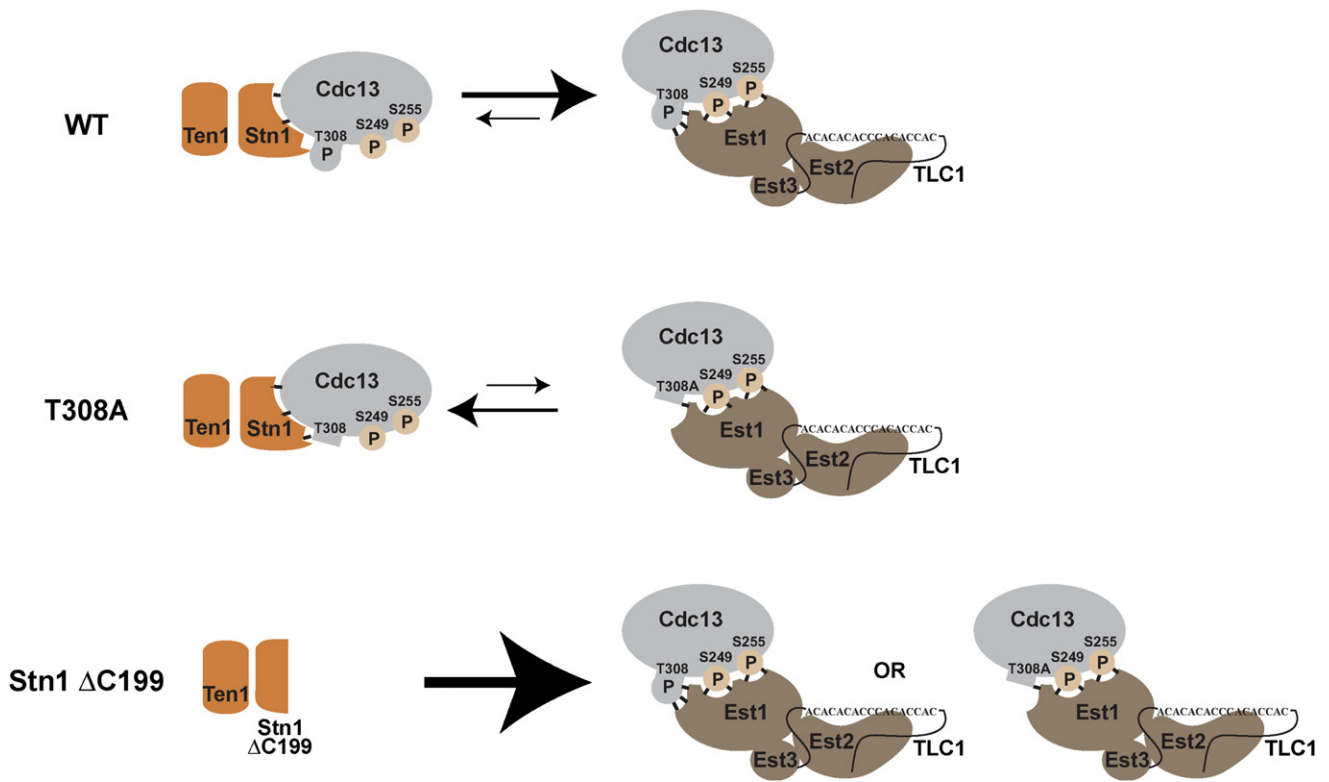
Figure 7. A Schematic Model for Potential Function of Cdc13 Threonine 308 Phosphorylation

To put these findings in context, we note that Cdk1 phosphorylation of Cdc13 at T308 will, in turn, need to be coordinated with other levels of Cdc13 regulation. In addition to Cdk1, the Tel1 and Mec1 kinases (homologs of mammalian ATM and ATR) have previously been shown to play important roles in telomere length maintenance. Tel1 and Mec1 control the DNA-damage response in budding yeast through phosphorylation of proteins involved in checkpoint control (such as Rad53) and DNA replication (such as RPA) (Brush et al., 1996; Sanchez et al., 1996). In budding yeast, in contrast to Mec1, Tel1 plays a minor role in the cellular response to DNA damage but a major role in telomere length maintenance. Loss of Tel1 results in telomere shortening (Lustig and Petes, 1986). While *tel1 mec1* double mutants have a senescence phenotype (Ritchie et al., 1999), they have normal telomerase enzymatic activity (Chan et al., 2001). Consistent with this observation, Tel1 and Mec1 are required for normal levels of association of Est1 and Est2 with telomeres, suggesting a potential role in promoting the recruitment of telomerase complex to telomeres (Goudsouzian et al., 2006). Recent studies also showed that Tel1 kinase activity is crucial for preferential recruitment of telomerase to a shortened telomere (Chang et al., 2007; Hector et al., 2007; Hirano and Sugimoto, 2007; Sabourin et al., 2007). Furthermore, Tel1 and Mec1 can phosphorylate Cdc13 at multiple sites in vitro. Single mutation of either of these in vitro phosphorylation sites (serine 249 and serine 255) to alanine resulted in telomere shortening, and double mutation of these two potential phosphoylation sites resulted in progressive telomere shortening and senescence in vivo (Tseng et al., 2006).

While combination of Cdc13 T308A with S249A and S255A mutations did not result in obvious synergistic telomere shortening (Figure S11), it is still conceivable that phosphorylation of Cdc13 by Cdk1 and by Tel1 and/or Mec1 may act synergistically to determine which telomere is elongated through preferential recruitment of a functional telomerase complex in late S to G2 phases. Further identification of potential Tel1/Mec1-dependent phosphorylation sites in Cdc13 and confirmation of these Tel1/Mec1 phosphorylation events in vivo using phospho-specific antibodies will be important to probe their potential roles in telomerase action on telomeres.

Based on the results reported here and on previous data, we propose the following model: cell-cycle progression into late S phase results in telomere end processing and elongation of the G-strand overhang. Cdk1 phosphorylates Cdc13 at T308. As depicted in Figure 7, the threonine 308 phosphorylated Cdc13 on the G-strand overhang results in preferential recruitment of the telomerase complex through its interaction with Est1 because the T308 phosphorylation biases the binding of Cdc13 toward Est1. Conversely, lack of T308 phosphorylation results in reduced interaction of Cdc13 and Est1, hence increasing the association of Cdc13 with the Stn1-Ten1 complex. Thus, this Cdk1-dependent phosphorylation of Cdc13 provides a mechanism that coordinates cell-cycle progression with the recruitment of telomerase relative to the Stn1-Ten1 complexes to telomeres in late S to G2. Deletion of the Stn1 C terminus results in loss of Cdc13 binding, hence alleviating negative telomere length regulation by the Stn1-Ten1 complex and resulting in

overelongation of telomeres. The results using the *stn1-ΔC199* mutant also argue against the *cdc13-T308A* mutation affecting the processing of single-strand G-rich overhangs. If *cdc13-T308A* caused an end processing phenotype, we should have seen a telomere length difference between *CDC13* and *cdc13-T308A* yeast strains in the *stn1-ΔC199* background. In addition to Cdk1-dependent phosphorylation, we suggest that the preferential recruitment of Tel1 to short telomeres and subsequent phosphorylation of Cdc13 (at serine 249 and serine 255 or other unidentified phosphorylation sites) by Tel1/Mec1 on the short telomere may preferentially synergize with the cdk1 phosphorylation to promote the recruitment of telomererase complex (likely through preferential binding to Est1) to that short telomere in a cell-cycle-dependent manner (Figure 7).

Previous data have shown that loss of Stn1 results in significant telomere elongation, while Stn1 overexpression results in telomere shortening (Chandra et al., 2001; Dahlseid et al., 2003; Grossi et al., 2004). It was therefore proposed that the interaction of the Stn1-Ten1 complex with Cdc13 could compete with the recruitment of telomerase complex by Cdc13, thereby inhibiting telomerase-dependent telomere addition (Chandra et al., 2001; Pennock et al., 2001). Consistent with this hypothesis, we found that the telomere shortening induced by loss of Cdk1-dependent phosphorylation of Cdc13 is fully alleviated in *stn1-ΔC199* yeast, in which the interaction of Stn1 and Cdc13 is disrupted. Hence, since Cdc13 can no longer interact with Stn1, phosphorylation of Cdc13 threonine 308 is no longer necessary to promote the competing interaction with Est1. Using ChIP analysis, we also detected recruitment of Stn1 to telomeres during late S to G2 phases of the cell cycle in Cdc13 or *cdc13-T308A* yeast. Such cell-cycle-dependent recruitment of Stn1 to telomeres was recently reported by others (Puglisi et al., 2008). Interestingly, the magnitude of Stn1 telomere binding is independent of telomere length (Puglisi et al., 2008). Our data also suggest that the interaction of both Cdc13-3HA and Cdc13-T308A-3HA with overexpressed 13myc-Est1 is stronger than with 13myc-Stn1, so that even with the Cdc13 threonine 308 to alanine mutation, Est1 can still recruit the telomerase complex in the presence of Stn1-Ten1 competition, albeit less efficiently. This can explain why we only see a moderate telomere shortening in *cdc13-T308A* yeast but not an ever shorter telomere (EST) phenotype or senescence.

It will also be of interest to find out whether similar regulatory mechanisms modulate telomerase action in human cells. Such regulatory mechanisms may provide new targets for potential cancer therapy and for anti-aging research.

EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmid Constructions
See Supplemental Data for details.

In Vitro Kinase Assays
Active Cdk1-as1-TAP/cyclin complexes were purified from *sic1Δ pGAL-cdc28-as1-TAP* yeast cells as previously described (Puig et al., 2001). His-tagged Cdc13, Ten1, Est1, and Est3 recombinant proteins were expressed and batch purified from Tuner (DE3) codon plus bacteria (Novagen) using Ni-NTA beads. In vitro kinase assays were performed as previously described (Ubersax et al., 2003).

Antibodies and Western Blot Analysis
Whole-cell extracts were prepared by bead beating in lysis buffer and were subjected to immunoprecipitation as described (Anderson et al., 2008). For western blot analysis, anti-Myc 9E10 antibody (Covance), anti-HA 3F10 (Roche), and affinity-purified rabbit anti-Cdc13 threonine 308 phospho-specific antibody (raised against phospho-peptide (Ac-CYIQSQ(pT)PERKTS-amide, QCB) were used.

Phosphatase Treatment of Yeast Lysates
Yeast extract from asynchronous cell cultures were prepared by bead beating in yeast lysis buffer without phosphatase inhibitor but with complete EDTA-free protease inhibitor tablet (Roche). For treatment with lambda protein phosphatase (Upstate), $MnCl_2$ (final 2 mM), DTT (final 1 mM), and lambda protein phosphatase (final 1000 U/ml) were added. For lambda protein phosphatase treatment with phosphatase inhibitor, NaF (final 50 mM), β-glycerol phosphate (final 50 mM), and Na_3VO_4 (final 1 mM) were added. The lysates were incubated at 37°C for 1 hr followed by immunoprecipitation and western analysis as described above.

Coimmunoprecipitation of *TLC1* RNA
Yeast lysates from asynchronous cultures were immunoprecipitated using anti-myc (9E10) antibodies with Dynabeads protein G beads (Invitrogen). Total RNA on the beads was purified using RNeasy mini kit (QIAGEN) followed by on-column DNAase digestion. The amount of TLC1 and Actin mRNA copurified were quantified using real-time QRT-PCR (Stratagene). The enrichment of TLC1 was expressed as the ratio of TLC1 amount coimmunoprecipitated in the presence of anti-Myc 9E10 antibody in samples versus lysate from nontagged control strain (background) after normalization to total input ($TLC1_{test\ IP}/TLC1_{test\ input}$)($TLC1_{no\ tag\ input}/TLC1_{no\ tag}$). The enrichment of Actin mRNA was also calculated in the same way and used as control. The results show an average of three experiments. Statistically significant differences in data sets were established by using a Student's t test. The error bars represent standard deviation.

Southern Blot Analysis and Telomere Length Measurement
Yeast genomic DNAs were digested with XhoI, separated by 0.8% agarose gel electrophoresis, and transferred to Hybond-XL membrane (Amersham). The blot was probed for telomeric $C_{1-3}A$ sequence with the ^{32}P-end-labeled oligo-nucleotide (TGTGGTGTGTGGGTGTGGTGT). The in-gel native hybridization was previously described (Dionne and Wellinger, 1996).

Chromatin Immunoprecipitation
ChIP analysis was performed as previously described (Taggart et al., 2002). PCR primers were specific for amplification of 114 base pair sequence at XII-L Y′ subtelomeric sequence (TEL) and 372 base pair ARO1 sequence (ARO1, as negative control). Enrichment of telomere sequence is expressed as the fold of Y′ subtelomeric DNA immunoprecipitated per reaction from tagged strain relative to that from the nontagged strain after normalized to total input ($TEL_{test\ IP}/TEL_{test\ input}$)($TEL_{no\ tag\ input}/TEL_{no\ tag}$). As a control, the enrichment of ARO1 sequence was quantified similarly. The results show an average of three experiments. Statistically significant differences in data sets were established by using a Student's t test. The error bars represent standard deviation.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, one table, and 11 figures and can be found with this article online at http://www.cell.com/S0092-8674(08)01502-X.

REFERENCES

Anderson, C.M., Korkin, D., Smith, D.L., Makovets, S., Seidel, J.J., Sali, A., and Blackburn, E.H. (2008). Tel2 mediates activation and localization of ATM/Tel1 kinase to a double-strand break. Genes Dev. 22, 854–859.

Askree, S.H., Yehuda, T., Smolikov, S., Gurevich, R., Hawk, J., Coker, C., Krauskopf, A., Kupiec, M., and McEachern, M.J. (2004). A genome-wide screen for Saccharomyces cerevisiae deletion mutants that affect telomere length. Proc. Natl. Acad. Sci. USA 101, 8658–8663.

Bishop, A.C., Ubersax, J.A., Petsch, D.T., Matheos, D.P., Gray, N.S., Blethrow, J., Shimizu, E., Tsien, J.Z., Schultz, P.G., Rose, M.D., et al. (2000). A chemical switch for inhibitor-sensitive alleles of any protein kinase. Nature 407, 395–401.

Blackburn, E.H. (2000). Telomere states and cell fates. Nature 408, 53–56.

Bourns, B.D., Alexander, M.K., Smith, A.M., and Zakian, V.A. (1998). Sir proteins, Rif proteins, and Cdc13p bind Saccharomyces telomeres in vivo. Mol. Cell. Biol. 18, 5600–5608.

Brush, G.S., Morrow, D.M., Hieter, P., and Kelly, T.J. (1996). The ATM homologue MEC1 is required for phosphorylation of replication protein A in yeast. Proc. Natl. Acad. Sci. USA 93, 15075–15080.

Chan, S.W., Chang, J., Prescott, J., and Blackburn, E.H. (2001). Altering telomere structure allows telomerase to act in yeast lacking ATM kinases. Curr. Biol. 11, 1240–1250.

Chandra, A., Hughes, T.R., Nugent, C.I., and Lundblad, V. (2001). Cdc13 both positively and negatively regulates telomere replication. Genes Dev. 15, 404–414.

Chang, M., Arneric, M., and Lingner, J. (2007). Telomerase repeat addition processivity is increased at critically short telomeres in a Tel1-dependent manner in Saccharomyces cerevisiae. Genes Dev. 21, 2485–2494.

Chen, J.L., and Greider, C.W. (2004). Telomerase RNA structure and function: implications for dyskeratosis congenita. Trends Biochem. Sci. 29, 183–192.

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

Dahlseid, J.N., Lew-Smith, J., Lelivelt, M.J., Enomoto, S., Ford, A., Desruisseaux, M., McClellan, M., Lue, N., Culbertson, M.R., and Berman, J. (2003). mRNAs encoding telomerase components and regulators are controlled by UPF genes in Saccharomyces cerevisiae. Eukaryot. Cell 2, 134–142.

Diede, S.J., and Gottschling, D.E. (1999). Telomerase-mediated telomere addition in vivo requires DNA primase and DNA polymerases alpha and delta. Cell 99, 723–733.

Dionne, I., and Wellinger, R.J. (1996). Cell cycle-regulated generation of single-stranded G-rich DNA in the absence of telomerase. Proc. Natl. Acad. Sci. USA 93, 13902–13907.

Evans, S.K., and Lundblad, V. (1999). Est1 and Cdc13 as comediators of telomerase access. Science 286, 117–120.

Frank, C.J., Hyde, M., and Greider, C.W. (2006). Regulation of telomere elongation by the cyclin-dependent kinase CDK1. Mol. Cell 24, 423–432.

Gatbonton, T., Imbesi, M., Nelson, M., Akey, J.M., Ruderfer, D.M., Kruglyak, L., Simon, J.A., and Bedalov, A. (2006). Telomere length as a quantitative trait: genome-wide survey and genetic mapping of telomere length-control genes in yeast. PLoS Genet. 2, e35. 10.1371/journal.pgen.0020035.

Goudsouzian, L.K., Tuzon, C.T., and Zakian, V.A. (2006). S. cerevisiae Tel1p and Mre11p are required for normal levels of Est1p and Est2p telomere association. Mol. Cell 24, 603–610.

Grandin, N., Damon, C., and Charbonneau, M. (2001). Ten1 functions in telomere end protection and length regulation in association with Stn1 and Cdc13. EMBO J. 20, 1173–1183.

Grossi, S., Puglisi, A., Dmitriev, P.V., Lopes, M., and Shore, D. (2004). Pol12, the B subunit of DNA polymerase alpha, functions in both telomere capping and length regulation. Genes Dev. 18, 992–1006.

Hector, R.E., Shtofman, R.L., Ray, A., Chen, B.R., Nyun, T., Berkner, K.L., and Runge, K.W. (2007). Tel1p preferentially associates with short telomeres to stimulate their elongation. Mol. Cell 27, 851–858.

Hirano, Y., and Sugimoto, K. (2007). Cdc13 telomere capping decreases Mec1 association but does not affect Tel1 association with DNA ends. Mol. Biol. Cell 18, 2026–2036.

Hsu, C.L., Chen, Y.S., Tsai, S.Y., Tu, P.J., Wang, M.J., and Lin, J.J. (2004). Interaction of Saccharomyces Cdc13p with Pol1p, Imp4p, Sir4p and Zds2p is involved in telomere replication, telomere maintenance and cell growth control. Nucleic Acids Res. 32, 511–521.

Hughes, T.R., Weilbaecher, R.G., Walterscheid, M., and Lundblad, V. (2000). Identification of the single-strand telomeric DNA binding domain of the Saccharomyces cerevisiae Cdc13 protein. Proc. Natl. Acad. Sci. USA 97, 6457–6462.

Laroche, T., Martin, S.G., Tsai-Pflugfelder, M., and Gasser, S.M. (2000). The dynamics of yeast telomeres and silencing proteins through the cell cycle. J. Struct. Biol. 129, 159–174.

Larrivee, M., LeBel, C., and Wellinger, R.J. (2004). The generation of proper constitutive G-tails on yeast telomeres is dependent on the MRX complex. Genes Dev. 18, 1391–1396.

Lendvay, T.S., Morris, D.K., Sah, J., Balasubramanian, B., and Lundblad, V. (1996). Senescence mutants of Saccharomyces cerevisiae with a defect in telomere replication identify three additional EST genes. Genetics 144, 1399–1412.

Levy, D.L., and Blackburn, E.H. (2004). Counting of Rif1p and Rif2p on Saccharomyces cerevisiae telomeres regulates telomere length. Mol. Cell. Biol. 24, 10857–10867.

Lin, J.J., and Zakian, V.A. (1996). The Saccharomyces CDC13 protein is a single-strand TG1–3 telomeric DNA-binding protein in vitro that affects telomere behavior in vivo. Proc. Natl. Acad. Sci. USA 93, 13760–13765.

Lustig, A.J., and Petes, T.D. (1986). Identification of yeast mutants with altered telomere structure. Proc. Natl. Acad. Sci. USA 83, 1398–1402.

Marcand, S., Gilson, E., and Shore, D. (1997). A protein-counting mechanism for telomere length regulation in yeast. Science 275, 986–990.

Marcand, S., Brevet, V., Mann, C., and Gilson, E. (2000). Cell cycle restriction of telomere elongation. Curr. Biol. 10, 487–490.

Nugent, C.I., Hughes, T.R., Lue, N.F., and Lundblad, V. (1996). Cdc13p: a single-strand telomeric DNA-binding protein with a dual role in yeast telomere maintenance. Science 274, 249–252.

Osterhage, J.L., Talley, J.M., and Friedman, K.L. (2006). Proteasome-dependent degradation of Est1p regulates the cell cycle-restricted assembly of telomerase in Saccharomyces cerevisiae. Nat. Struct. Mol. Biol. 13, 720–728.

Pennock, E., Buckley, K., and Lundblad, V. (2001). Cdc13 delivers separate complexes to the telomere for end protection and replication. Cell 104, 387–396.

Petreaca, R.C., Chiu, H.C., Eckelhoefer, H.A., Chuang, C., Xu, L., and Nugent, C.I. (2006). Chromosome end protection plasticity revealed by Stn1p and Ten1p bypass of Cdc13p. Nat. Cell Biol. 8, 748–755.

Petreaca, R.C., Chiu, H.C., and Nugent, C.I. (2007). The role of Stn1p in Saccharomyces cerevisiae telomere capping can be separated from its interaction with Cdc13p. Genetics 177, 1459–1474.

Puglisi, A., Bianchi, A., Lemmens, L., Damay, P., and Shore, D. (2008). Distinct roles for yeast Stn1 in telomere capping and telomerase inhibition. EMBO J. 27, 2328–2339.

Puig, O., Caspary, F., Rigaut, G., Rutz, B., Bouveret, E., Bragado-Nilsson, E., Wilm, M., and Seraphin, B. (2001). The tandem affinity purification (TAP) method: a general procedure of protein complex purification. Methods 24, 218–229.

Ritchie, K.B., Mallory, J.C., and Petes, T.D. (1999). Interactions of TLC1 (which encodes the RNA subunit of telomerase), TEL1, and MEC1 in regulating telomere length in the yeast Saccharomyces cerevisiae. Mol. Cell. Biol. *19*, 6065–6075.

Sabourin, M., Tuzon, C.T., and Zakian, V.A. (2007). Telomerase and Tel1p preferentially associate with short telomeres in *S. cerevisiae*. Mol. Cell *27*, 550–561.

Sanchez, Y., Desany, B.A., Jones, W.J., Liu, Q., Wang, B., and Elledge, S.J. (1996). Regulation of RAD53 by the ATM-like kinases MEC1 and TEL1 in yeast cell cycle checkpoint pathways. Science *271*, 357–360.

Schramke, V., Luciano, P., Brevet, V., Guillot, S., Corda, Y., Longhese, M.P., Gilson, E., and Geli, V. (2004). RPA regulates telomerase action by providing Est1p access to chromosome ends. Nat. Genet. *36*, 46–54.

Smith, C.D., Smith, D.L., DeRisi, J.L., and Blackburn, E.H. (2003). Telomeric protein distributions and remodeling through the cell cycle in Saccharomyces cerevisiae. Mol. Biol. Cell *14*, 556–570.

Taggart, A.K., Teng, S.C., and Zakian, V.A. (2002). Est1p as a cell cycle-regulated activator of telomere-bound telomerase. Science *297*, 1023–1026.

Tseng, S.F., Lin, J.J., and Teng, S.C. (2006). The telomerase-recruitment domain of the telomere binding protein Cdc13 is regulated by Mec1p/Tel1p-dependent phosphorylation. Nucleic Acids Res. *34*, 6327–6336.

Ubersax, J.A., Woodbury, E.L., Quang, P.N., Paraz, M., Blethrow, J.D., Shah, K., Shokat, K.M., and Morgan, D.O. (2003). Targets of the cyclin-dependent kinase Cdk1. Nature *425*, 859–864.

Vodenicharov, M.D., and Wellinger, R.J. (2006). DNA degradation at unprotected telomeres in yeast is regulated by the CDK1 (Cdc28/Clb) cell-cycle kinase. Mol. Cell *24*, 127–137.

Wellinger, R.J., Wolf, A.J., and Zakian, V.A. (1993). *Saccharomyces* telomeres acquire single-strand TG1–3 tails late in S phase. Cell *72*, 51–60.

Wright, J.H., Gottschling, D.E., and Zakian, V.A. (1992). Saccharomyces telomeres assume a non-nucleosomal chromatin structure. Genes Dev. *6*, 197–210.

Wright, J.H., and Zakian, V.A. (1995). Protein-DNA interactions in soluble telosomes from Saccharomyces cerevisiae. Nucleic Acids Res. *23*, 1454–1460.

Zakian, V.A. (1996). Structure, function, and replication of Saccharomyces cerevisiae telomeres. Annu. Rev. Genet. *30*, 141–172.