Telomerase Recruitment in *Saccharomyces cerevisiae* Is Not Dependent on Tel1-Mediated Phosphorylation of Cdc13

Hua Gao,^{*,†,1} Tasha B. Toro,^{*,‡,2} Margherita Paschini,^{*,§} Bari Braunstein-Ballew,^{*,§}
Rachel B. Cervantes^{*} and Victoria Lundblad^{*,3}

^{*}Salk Institute for Biological Studies, La Jolla, California 92037-1099, [†]Graduate Program in Biochemistry and Molecular Biology and [‡]Graduate Program in Molecular and Human Genetics, Baylor College of Medicine, Houston Texas 77030 and [§]Division of Biological Sciences, University of California San Diego, La Jolla, California 92093-0130

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ABSTRACT

In *Saccharomyces cerevisiae*, association between the *Est1* telomerase subunit and the telomere-binding protein *Cdc13* is essential for telomerase to be recruited to its site of action. A current model proposes that *Tel1* binding to telomeres marks them for elongation, as the result of phosphorylation of a proposed S/TQ cluster in the telomerase recruitment domain of *Cdc13*. However, three observations presented here argue against one key aspect of this model. First, the pattern of *Cdc13* phosphatase-sensitive isoforms is not altered by loss of *Tel1* function or by mutations introduced into two conserved serines (S249 and S255) in the *Cdc13* recruitment domain. Second, an interaction between *Cdc13* and *Est1*, as monitored by a two-hybrid assay, is dependent on S255 but *Tel1*-independent. Finally, a derivative of *Cdc13*, *cdc13*-(S/TQ)₁₁→(S/TA)₁₁, in which every potential consensus phosphorylation site for *Tel1* has been eliminated, confers nearly wild-type telomere length. These results are inconsistent with a model in which the *Cdc13*–*Est1* interaction is regulated by *Tel1*-mediated phosphorylation of the *Cdc13* telomerase recruitment domain. We propose an alternative model for the role of *Tel1* in telomere homeostasis, which is based on the assumption that *Tel1* performs the same molecular task at double-strand breaks (DSBs) and chromosome termini.

TELOMERE length homeostasis is a highly regulated process that must balance telomere loss (as the result of incomplete replication and/or nucleolytic degradation) with telomeric repeat addition (through the action of telomerase and/or recombination). In the budding yeast *Saccharomyces cerevisiae*, a key regulatory event is recruitment of telomerase to chromosome ends by the telomere end-binding protein *Cdc13* (NUGENT *et al.* 1996; EVANS and LUNDBLAD 1999; PENNOCK *et al.* 2001; BIANCHI *et al.* 2004; CHAN *et al.* 2008). Recruitment relies on a direct interaction between *Cdc13* and the *Est1* subunit of telomerase (PENNOCK *et al.* 2001), which brings the catalytic core of the enzyme to its site of action. Disruption of this interaction, due to mutations in either *CDL13* (*cdc13-2*) or *EST1* (*est1-60*), results in an Est (ever-shorter-telomere) phenotype, as manifested by progressive telo-

mere shortening and an eventual senescence phenotype. The recruitment activity of *Cdc13*, which resides in a 15-kDa N-terminal domain (PENNOCK *et al.* 2001), is sufficient to direct telomerase even to nontelomeric sites (BIANCHI *et al.* 2004). As predicted by the recruitment model, association of telomerase with telomeres is greatly reduced in strains expressing the recruitment-defective *cdc13-2* allele (CHAN *et al.* 2008).

Telomerase action at individual telomeres is highly regulated. Using an assay that monitors telomere addition at single nucleotide resolution (single telomere extension, STE), LINGNER and colleagues showed that only ~7% of telomeres with wild-type (*i.e.*, 300 bp) length are elongated by telomerase during a single cell cycle (TEIXEIRA *et al.* 2004). However, as telomere length declines, the extension frequency increases: ~20% of telomeres 200 bp in length and >40% of 100-bp-long telomeres are elongated (TEIXEIRA *et al.* 2004; ARNERIC and LINGNER 2007). The mechanism by which telomerase distinguishes short from long telomeres has been the subject of intense investigation. Work from a number of laboratories has led to the proposal that *Tel1*-dependent phosphorylation of *Cdc13* at under-elongated telomeres mediates the interaction between *Cdc13* and the telomerase-associated *Est1* protein, thus ensuring that telomerase is directed to the shortest

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¹Present address: Department of Cell Biology, University of Texas Southwestern Medical Center, Dallas, TX 75390-9039.

²Present address: Sanford-Burnham Medical Research Institute, La Jolla, CA 92037.

³Corresponding author: Salk Institute for Biological Studies, 10010 N. Torrey Pines Rd., La Jolla, CA 92037-1099. E-mail: lundblad@salk.edu

telomeres in a population. In support of this model, the *Est1* and *Est2* telomerase subunits exhibit enhanced association with telomeres that have been artificially shortened, whereas *Cdc13* displays length-independent association with telomeres (BIANCHI and SHORE 2007; SABOURIN *et al.* 2007). This suggests that the preferential elongation of shorter telomeres is controlled at the level of recruitment of the telomerase holoenzyme by *Cdc13*. Furthermore, efficient association of *Est1* and *Est2* with chromosome ends requires *Tell* and *Mre11* (which acts in the same pathway as *Tell* for telomere length regulation; NUGENT *et al.* 1998; RITCHIE and PETES 2000) but not *Mec1* (TAKATA *et al.* 2005; GOUDSOUZIAN *et al.* 2006). *Tell* itself is also telomere bound (TAKATA *et al.* 2004), with enhanced binding to shorter telomeres (BIANCHI and SHORE 2007; HECTOR *et al.* 2007; SABOURIN *et al.* 2007; ABDALLAH *et al.* 2009), although there is considerable controversy over the degree and timing of *Tell* association with chromosome termini during the cell cycle. As expected for a key regulator of the interaction between *Cdc13* and a telomerase subunit, a *tell*- Δ strain has short telomeres (LUSTIG and PETES 1986), although telomere length is not impaired enough to confer the Est phenotype displayed by *cdc13-2* and *est1-60* strains.

Implicit in the above proposal is that *Cdc13* must be a direct substrate of *Tell*. In support of this, Teng and colleagues reported several years ago that the recruitment domain of *Cdc13* has a cluster of potential *Tell* (and/or *Mec1*) phosphorylation sites (TSENG *et al.* 2006). Substrates of the DNA damage kinases often contain several closely spaced phosphorylation sites, termed S/TQ cluster domains (SCDs), which are considered a structural hallmark of many DNA damage-response proteins (TRAVERN and HEIERHORST 2005). On the basis of *in vitro* kinase assays with GST fusions to 75- to 90-amino-acid portions of the *Cdc13* recruitment domain, TSENG *et al.* 2006 concluded that four SQ sites in the recruitment domain of *Cdc13* are overlapping substrates for both *Tell* and *Mec1*, leading to the proposal that telomerase recruitment in *S. cerevisiae* is regulated by *Tell*-dependent phosphorylation of *Cdc13*.

The above model makes a key prediction: in a *tell*- Δ strain, telomerase should no longer exhibit a length-dependent pattern of elongation. However, preferential elongation of short telomeres still occurs at native chromosome ends in the absence of *Tell* (ARNERIC and LINGNER 2007). In addition, PETES and colleagues have argued, on the basis of epistasis data, that *Tell* performs an indirect role in the telomerase pathway, rather than directly targeting a telomerase regulator (RITCHIE *et al.* 1999; RITCHIE and PETES 2000). These observations are not easily explained, if preferential recognition of short telomeres by telomerase is mediated by *Tell*-dependent phosphorylation of *Cdc13*. In this current study, we have re-examined the evidence for phosphorylation of *Cdc13* as a regulatory mechanism

for telomere length homeostasis. We report on a series of observations that indicate that *Tell* contributes to telomere length control through a mechanism other than phosphorylation of the *Cdc13* S/TQ cluster.

MATERIALS AND METHODS

Strains and plasmids: The yeast strains and plasmids used in this study are shown in Tables 1 and 2, respectively. All plasmids containing missense mutations were generated by QuickChange mutagenesis, and a sequenced restriction fragment was subcloned back into an unmutagenized backbone, to eliminate the possibility of unlinked mutations. The diploid strains used in Figure 7 were constructed by introducing the relevant mutation into a haploid strain, using the *URA3* integrating constructs listed in Table 2, and selecting for “pop-outs” on 5-FOA-containing media. The genotype of the *CDC13* or *TEL2* locus in the resulting 5-FOA-resistant strains was determined by molecular diagnostics as well as by telomere length, and mutant haploids were subsequently mated to an isogenic *tlc1*- Δ haploid. The resulting heterozygous diploid strains were completely isogenic, with the exception of the relevant loci (*TLC1*, *CDC13*, *TEL1*, or *TEL2*).

Genetic methods: Telomere length and senescence phenotypes were analyzed following dissection of strains bearing mutations integrated into the genome or as plasmid-borne mutations transformed into a *cdc13*- Δ /p *URA3 CDC13* shuffle strain. In the latter case, transformants were streaked for single colonies on 5-FOA-containing media to identify isolates that had lost the covering *CDC13* plasmid and subsequently monitored for the appearance of a senescence phenotype on successive streak-outs, as described previously (LUNDBLAD and SZOSTAK 1989; LENDVAY *et al.* 1996). For the epistasis analysis shown in Figure 7, senescence was assayed through a genotype-blind assessment of the growth characteristics of multiple isolates of a *tlc1*- Δ strain, compared to multiple isolates of a *tlc1*- Δ strain bearing a second mutation (RIZKI and LUNDBLAD 2001). Following dissection of the relevant diploid, haploid spores containing the *tlc1*- Δ mutation were identified and subsequently streaked for single colonies three successive times. After 48 hr incubation at 30°, growth was scored on a scale of 1 (severe senescence) to 5 (comparable to wild-type growth), and the *CDC13*, *TEL1*, or *TEL2* genotype of each haploid strain was determined at the conclusion of the experiment. Data for each genotype at each of the three time points were displayed on a histogram, and a Student's *t*-test was used to assess the statistical significance between genotypes at each time point.

De novo telomere assays were performed as described previously (BIANCHI *et al.* 2004), with several slight modifications: strains expressing the GBD-*Cdc13*-RD protein fusions were grown in liquid selective media containing 2% raffinose, galactose was added to a final concentration of 3% for 3.5 hr, and serial dilutions were plated and allowed to grow for 2 days to form visible colonies. Colonies were replica plated onto plates containing 5-FOA or α -aminoadipate to detect loss of the distal *URA3* or *LYS2* genes, respectively.

Biochemical analysis: Protease-deficient yeast strains (either *TEL1* or *tell*- Δ) expressing *Ccd13*-(myc)₁₈ were grown in selective media to OD₆₀₀ = 0.8. Washed pellets were resuspended in cold TMG200 (10 mM Tris-HCl pH 8.0, 1 mM MgCl₂, 10% glycerol, 200 mM NaCl), lysed using a mortar and pestle in the presence of liquid N₂, and extracts were clarified by spinning two times at 14,000 rpm for 20 min. For phosphatase-treated samples, 80 μ g of protein lysate was incubated at 30° for 1 hr with 80 units of λ -phosphatase (NEB) in 50 mM

TABLE 1
Strains used in this study

Strain	Genotype
YVL2540	<i>MATa cdc13-Δ::LYS2 ura3-52 lys2-801 trp1-Δ1 his3-Δ200 leu2-Δ1 ade2-101/pVL438</i>
YVL3006	<i>MATα cdc13-Δ::LYS2 ura3-52 lys2-801 trp1-Δ1 his3-Δ200 leu2-Δ1/pVL438</i>
YVL2985 ^a	<i>MATa/α tlc1-Δ::HIS3/TLC1 tel2-1/TEL2</i>
YVL2993 ^a	<i>MATa/α tlc1-Δ::HIS3/TLC1 cdc13-2/CDC13</i>
YVL3007 ^a	<i>MATa/α tlc1-Δ::HIS3/TLC1 cdc13-S249A,S255A/CDC13</i>
YVL3009 ^a	<i>MATa/α tlc1-Δ::HIS3/TLC1 cdc13-S255A/CDC13</i>
YVL3015 ^a	<i>MATa/α tlc1-Δ::HIS3/TLC1 tel1-Δ::KANMX6/TEL1</i>
YAB208	<i>mat-Δ ade2-Δ LEU2::GAL-HO lys2-Δ mnt2-Δ::LYS2 adh4-Δ::IM-3 rad52-Δ::SpHIS5 (ADE2-4XUASg-HO site-URA3)</i>
PJ69-4A	<i>MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4-Δ gal80-Δ GAL2-ADE2 LYS2::GAL1-HIS3 met2::GAL7-lacZ</i>
YVL2929	<i>MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4-Δ gal80-Δ GAL2-ADE2 LYS2::GAL1-HIS3 met2::GAL7-lacZ tel1-Δ::KANMX6</i>
YVL3052	<i>MATa leu2 trp1 ura3-52 prb⁻ prc⁻ pep4-3 sml1-Δ::NAT</i>
YVL3053	<i>MATa leu2 trp1 ura3-52 prb⁻ prc⁻ pep4-3 sml1-Δ::NAT mec1-Δ::TRP1</i>
YVL3054	<i>MATa leu2 trp1 ura3-52 prb⁻ prc⁻ pep4-3 sml1-Δ::NAT tel1-Δ::KANMX6</i>

^a Additional genotype: *ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 trp1-Δ1/trp1-Δ1 his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1*.

HEPES pH 7.5, 100 mM NaCl, 2 mM DTT, 0.01% Brij 35, 1 mM MgCl₂ and clarified by spinning at 14,000 rpm for 10 min. Samples were resolved on 6% PAGE (30:0.39 acrylamide:bis) and subjected to Western analysis with 9E10 anti-myc antibody.

RESULTS

Analysis of mutations in two conserved serines in the recruitment domain of Cdc13: The *S. cerevisiae* Cdc13 protein contains 11 potential consensus phosphorylation sites for the Tel1 and/or Mec1 kinases; four of these motifs (S₂₂₅Q, S₂₄₉Q, S₂₅₅Q, and S₃₀₆Q) reside in the telomerase recruitment domain of Cdc13 and have been proposed to form an S/TQ cluster domain (TSENG *et al.* 2006). To evaluate the potential role of phosphorylation in recruitment of telomerase by Cdc13, we constructed an alignment of full-length Cdc13 proteins from a dozen fungal species belonging to the Saccharomycetes clade. Examination of this alignment revealed that only 2 of the 11 S/TQ motifs (S₂₄₉Q and S₂₅₅Q) in the *S. cerevisiae* Cdc13 protein were highly conserved (Figure 1A and data not shown). The S₂₅₅Q motif was invariant among the entire collection of 12 species, and the S₂₄₉Q motif was similarly conserved (although not invariant). In contrast, the remaining 9 SQ or TQ motifs were not conserved, including the 2 other members of the proposed S/TQ cluster domain (S₂₂₅Q and S₃₀₆Q), even among species within the *Saccharomyces sensu stricto* group (Figure 1A).

The two potential phosphorylation sites that were highly conserved (S₂₄₉Q and S₂₅₅Q) bracket residue E252 (Figure 1A), which has been previously shown to be essential for the interaction between Cdc13 and Est1 to mediate recruitment of telomerase to chromosome ends (EVANS and LUNDBLAD 1999; PENNOCK *et al.* 2001; BIANCHI *et al.* 2004). We therefore compared alleles of

CDC13, which replaced either S255 or S249 with alanine for their effects on telomere replication with the previously well-characterized *cdc13-2* mutation (bearing a charge swap mutation at residue E252). The *cdc13-S255A* strain displayed a pronounced telomere replication defect, as evidenced by a senescence phenotype (although the appearance of senescence was delayed, relative to the *cdc13-2* strain) and extremely short telomeres (Figure 1, B and C and [supporting information, Figure S1](#)). Furthermore, continued propagation of the *cdc13-S255A* strain gave rise to telomeric rearrangements (Figure S1), which are characteristic of a recombination-based pathway for telomere maintenance that can be employed in the absence of telomerase. In contrast, the *cdc13-S249A* strain displayed only a modest decline in telomere length, with no accompanying senescence (Figure 1, B and C), indicating that residue S249 makes a more minimal contribution to telomere length regulation.

The effects of mutations in S249 and S255 were also examined in a *de novo* telomere healing assay in which telomerase is ectopically recruited to a nontelomeric DNA end (BIANCHI *et al.* 2004). In this assay, the recruitment domain of Cdc13 (Cdc13-RD) is placed adjacent to a newly created double-strand break (DSB), as a GBD-Cdc13-RD fusion protein, which is capable of binding to a Gal4 DNA binding site located next to an HO-induced DSB. Telomere healing was detected by monitoring the loss of either of two genes, *URA3* or *LYS2*, which are distal to the HO-induced break. As shown in Figure 2, the GBD-Cdc13-RD-S255A fusion was incapable of mediating telomere healing following induction of a DSB. In cells expressing this fusion, very few colonies were able to grow following HO induction on media that detected loss of the distal *URA3* or *LYS2* genes (Figure 2 and data not shown), compared to the

TABLE 2
Plasmids used in this study

Plasmid	Description	Source
pVL438	<i>CEN URA3 CDC13</i>	EVANS and LUNDBLAD (1999)
pVL648	<i>CEN LEU2 CDC13</i>	EVANS and LUNDBLAD (1999)
pVL1084	<i>CEN LEU2 CDC13</i>	PENNOCK <i>et al.</i> (2001)
pVL690	<i>CEN LEU2 cdc13-E252K (cdc13-2)</i>	PENNOCK <i>et al.</i> (2001)
pVL1519	<i>CEN LEU2 cdc13-S255A</i>	This work
pVL3512	<i>CEN LEU2 cdc13-P235A</i>	This work
pVL3516	<i>CEN LEU2 cdc13-F237A</i>	This work
pVL1521	<i>CEN LEU2 cdc13-S249A</i>	This work
pVL2570	<i>CEN LEU2 cdc13-S249A</i>	This work
pVL3518	<i>CEN LEU2 cdc13-Q250A</i>	This work
pVL3513	<i>CEN LEU2 cdc13-N251A</i>	This work
pVL1727	<i>CEN LEU2 cdc13-E252A</i>	This work
pVL3515	<i>CEN LEU2 cdc13-F253A</i>	This work
pVL3517	<i>CEN LEU2 cdc13-N254A</i>	This work
pVL2571	<i>CEN LEU2 cdc13-S255A</i>	This work
pVL4351	<i>CEN LEU2 cdc13-Q256A</i>	This work
pVL3335	<i>CEN LEU2 cdc13-S306A</i>	This work
pVL4140	<i>CEN LEU2 cdc13-4Q→4A^a</i>	This work
pVL5005	<i>CEN LEU2 cdc13-11Q→11A^b</i>	This work
pVL2691	<i>URA3 cdc13-2</i> (integrating vector)	This work
pVL2619	<i>URA3 cdc13-S255A</i> (integrating vector)	This work
pVL2693	<i>URA3 cdc13-S249A, S255A</i> (integrating vector)	This work
pVL2859	<i>URA3 tel2-1</i> (integrating vector)	This work
pVL3277	<i>2μ LEU2 CDC13-(myc)₁₈</i>	This work
pVL2640	<i>2μ LEU2 cdc13-S249A, S255A-(myc)₁₈</i>	This work
pAB301	<i>CEN TRP1 ADH-GBD-CDC13-RD^{aa211-331}</i>	BIANCHI <i>et al.</i> (2004)
pAB313	<i>CEN TRP1 ADH-GBD-CDC13-RD^{aa211-331}-E252K</i>	BIANCHI <i>et al.</i> (2004)
pVL3068	<i>CEN TRP1 ADH-GBD-CDC13-RD^{aa211-331}-S255A</i>	This work
pVL3070	<i>CEN TRP1 ADH-GBD-CDC13-RD^{aa211-331}-S249A</i>	This work
pVL693	<i>2μ LEU2 ADH-GAL4-AD-EST1</i>	This work
pVL859	<i>2μ LEU2 ADH-GAL4-AD-STN1^{aa5-494}</i>	CHANDRA <i>et al.</i> (2001)
pVL3125	<i>2μ TRP1 ADH-GAL4-DBD-CDC13^{R635A}</i>	GAO <i>et al.</i> (2007)
pVL3346	<i>2μ TRP1 ADH-GAL4-DBD-CDC13^{R635A E252K}</i>	This work
pVL3345	<i>2μ TRP1 ADH-GAL4-DBD-CDC13^{R635A S255A}</i>	This work
pVL3484	<i>2μ TRP1 ADH-GAL4-DBD-CDC13^{R635A S249A}</i>	This work
pVL3527	<i>2μ TRP1 ADH-GAL4-DBD-CDC13^{R635A P235S}</i>	This work
pVL3524	<i>2μ TRP1 ADH-GAL4-DBD-CDC13^{R635A F237V}</i>	This work
pVL3525	<i>2μ TRP1 ADH-GAL4-DBD-CDC13^{R635A F253A}</i>	This work
pVL3526	<i>2μ TRP1 ADH-GAL4-DBD-CDC13^{R635A N254A}</i>	This work

^a 4Q→4A: cdc13-Q226A, Q250A, Q256A, Q307A.

^b 11Q→11A: cdc13-Q66A, Q226A, Q250A, Q256A, Q307A, Q391A, Q476A, Q612A, Q644A, Q653A, Q828A.

efficient healing observed with the wild-type GBD-Cdc13-RD fusion. As expected, *de novo* telomere formation was also defective in the presence of the GBD-Cdc13-RD-E252K fusion (referred to as GBD-Cdc13-RD^{est} in BIANCHI *et al.* 2004). In contrast, telomere healing occurred at roughly wild-type levels with a GBD-Cdc13-RD-S249A fusion (Figure 2), which parallels the minimal effect that this allele had on telomere function at native chromosome termini.

A two-hybrid interaction between Cdc13 and Est1 is dependent on the recruitment domain of Cdc13, but does not require Tell1: The above analysis demonstrates that residue S255, like E252, is crucial for telomere replication, and the proximity of these two residues

suggests that S255 also mediates telomerase recruitment by contributing to the Cdc13-Est1 interaction. Since the *in vivo* experiments shown in Figures 1 and 2 do not directly test this interaction, we turned to an alternative means of examining whether the association between Est1 and the mutant Cdc13-S255A protein was impaired. In previous efforts by our lab and others, it has not been possible to detect an interaction between Cdc13 and Est1 using either coimmunoprecipitation from yeast extracts under endogenous expression conditions or with purified proteins *in vitro*. This precluded a direct biochemical examination of the Cdc13-S255A mutant protein. Therefore, we developed a two-hybrid assay to probe the Cdc13-Est1 interaction, using the

FIGURE 2.—Residue S255, but not S249, is required in a *de novo* telomere addition assay. Strains expressing the indicated GBD-Cdc13-RD protein fusions were grown in selective media containing 2% raffinose, exposed to 3% galactose for 3.5 hr to induce cleavage by the HO endonuclease to generate a double-strand break adjacent to four copies of the Gal4 DNA binding site, and aliquots were plated for single colonies. Plates were incubated for 3 days at 30° to allow colony growth and subsequently replica plated onto media that only allows growth of viable Ura^{-} cells, which requires healing of the HO-induced break by conversion into a functional telomere; the Ura^{-} phenotype is due to loss of the *URA3*-containing distal portion of the chromosome.

full-length Cdc13 and Est1 proteins fused to Gal4DBD or Gal4AD, respectively (see Figure S2 for a comparison of these reagents with a previously published set of Cdc13 and Est1 two-hybrid constructs used by Qi and ZAKIAN 2000). In this assay, a weak interaction between Cdc13 and Est1 could be observed, as exhibited by the ability to activate the His reporter but not the more stringent Ade reporter (Figure 3 and data not shown). As predicted, the Cdc13-Est1 association was disrupted by the *cdc13-2* (E252K) and *est1-60* mutations, arguing that this assay was probing a biologically valid interaction (Figure 3A and data not shown). The Gal4DBD-Cdc13-S255A construct was also defective for interaction with Est1, indicating that the telomere replication defect of the *cdc13-S255A* strain was due to a reduced interaction with telomerase. In contrast, the *cdc13-S249A* mutation, which only made a minor contribution to telomere length (Figure 1), had little effect in this two-hybrid test. Neither the *cdc13-2* nor the *cdc13-S255A* mutations altered the interaction with Stn1 in this assay

(Figure 3A), indicating that the inability to interact with Est1 was not due to nonspecific destabilization of the Gal4DBD-Cdc13-E252K or Gal4DBD-Cdc13-S255A constructs. The correlation between *in vivo* telomere length maintenance and a two-hybrid interaction between Cdc13 and Est1 was further supported by the behavior of additional mutations in the recruitment domain of Cdc13, as discussed in a later section.

This two-hybrid assay also provided a means of evaluating the potential role of Tel1 in mediating the interaction between Cdc13 and Est1, by comparing the behavior of *TEL1* and *tel1-Δ* strains in this test. As shown in Figure 3B, an interaction between Cdc13 and Est1 could still be observed in a *tel1-Δ* strain. Furthermore, this Cdc13-Est1 interaction was still mediated by the Cdc13 recruitment domain, as it was abolished by the *cdc13-2* mutation, similar to the behavior in the *TEL1* strain. These results indicate that the Cdc13-Est1 two-hybrid interaction was dependent on the serine residue at position 255, but not dependent on Tel1 function.

We also attempted to ask whether this two-hybrid interaction could be maintained in the absence of both Tel1 and Mec1. Unexpectedly, the two-hybrid strain was substantially more sensitive than other strain backgrounds to the simultaneous loss of both Tel1 and Mec1 function, as a *mec1-Δ tel1-Δ sml1-Δ* derivative of this strain was essentially inviable after propagation for ~25 generations (Figure S2), which precluded a test of the potential two-hybrid interaction between Cdc13 and Est1 in the absence of both kinases. Therefore, we cannot rule out the possibility that phosphorylation by Mec1 in the absence of Tel1 contributes to the Cdc13-Est1 interaction. However, it is worth pointing out that strains expressing kinase-dead or null alleles of *MEC1* do not display a defect in telomere elongation (MALLORY and PETES 2000; FRANK *et al.* 2006), indicating that Mec1 does not contribute to telomerase recruitment *in vivo*.

The phosphatase-sensitive mobility of Cdc13 is not affected by mutations in the recruitment domain or by loss of Tel1 or Mec1 function: To monitor phosphorylation, the mobility of Cdc13 bearing a C-terminal (myc)₁₈ epitope was examined on 6% SDS-PAGE gels using an altered acrylamide-bis ratio to enhance separation of potential phospho-isoforms. Under these conditions, the Cdc13-(myc)₁₈ protein exhibited an altered mobility, which was sensitive to λ-phosphatase (Figure 4A). This provided a reagent for testing whether Mec1- and/or Tel1-mediated phosphorylation occurs at either S₂₄₉Q or S₂₅₅Q, as previously proposed, by examining the mobility of the Cdc13-(myc)₁₈ protein bearing mutations in both S249 and S255. As shown in Figure 4A, no alteration in the pattern of the phosphatase-sensitive isoform(s) of the Cdc13-(myc)₁₈-S249A, S255A protein was observed. Furthermore, the altered mobility exhibited by the Cdc13-(myc)₁₈ protein was unchanged in a *tel1-Δ* strain (Figure 4B) or in a *tel1-Δ mec1-Δ* strain (Figure S3). Although these observations are subject to

FIGURE 3.—A two-hybrid interaction between Cdc13 and Est1 is dependent on S255 but not on Tel1. (A) Two-hybrid analysis of the interaction between Cdc13–R635A (wild-type or mutant versions of pVL3125) and either Est1 (top) or Stn1 (bottom); dilutions of the *S. cerevisiae* strain pJ69-4A, coexpressing Gal4DBD–Cdc13 and either Gal4AD–Est1 or Gal4AD–Stn1, were plated on appropriate selective plates to monitor viability and the ability to activate either the *GAL1–HIS3* or *GAL2–ADE2* reporter genes, respectively. The Gal4AD–Est1 fusion protein complemented an *est1*–Δ strain (data not shown). The R635A mutation in the DNA binding domain of Cdc13, which eliminates DNA binding (ANDERSON *et al.* 2003), was necessary to alleviate the lethality due to overexpression of Cdc13. (B) Two-hybrid analysis of the interaction between Gal4DBD–Cdc13 or Gal4DBD–Cdc13-2 (E252K) and Gal4AD–Est1 in pJ69-4A *vs.* an isogenic *tel1*–Δ derivative of pJ69-4A, performed as in A.

the caveat that not all phosphorylation events result in a mobility shift, our assay conditions nevertheless were sensitive enough to detect a change in mobility when a previously described Cdk phosphorylation site in Cdc13 at T₃₀₈P (LI *et al.* 2009) was eliminated (Figure S3).

As a more direct test of the phosphorylation status of S255, we raised phospho-specific antibodies that specifically recognized the proposed phospho-isoform of S255 *in vitro*, as assessed using an affinity-purified domain of Cdc13 containing S255 (see Figure S4 for more details). This antibody preparation failed to recognize S255-specific phosphorylation of Cdc13–(myc)₁₈ protein expressed in yeast (data not shown), even though immunoprecipitated Cdc13 protein was examined to maximize detection of S255 phosphorylation; nevertheless, we cannot exclude the possibility that this negative result was a consequence of the detection limits of this particular antibody. Collectively, these attempts to obtain direct evidence that S249 or S255 were phosphorylated *in vivo* were unsuccessful.

A variant of Cdc13 lacking all 11 SQ or TQ phosphorylation consensus sites confers wild-type telomere length: The above results raised the possibility that S255 and S249 might perform a structural role in telomerase recruitment, rather than as targets for a kinase. To differentiate between these two possibilities, we asked whether the adjacent glutamine residues in

the S₂₄₉Q and S₂₅₅Q motifs might be dispensable for *in vivo* function, by mutating Q250 and Q256 to alanine residues. Several studies have demonstrated that a glutamine amino acid in the +1 position relative to the targeted serine is critical for substrate recognition by the DNA damage kinases (KIM *et al.* 1999; SMOLKA *et al.* 2007; CHEN *et al.* 2010). Thus, if S₂₄₉Q and S₂₅₅Q are in fact targets for phosphorylation by Tel1 (or Mec1), this predicts that the *cdc13*–Q250A and *cdc13*–Q256A mutant strains should exhibit defects in telomere length regulation similar to that of the *cdc13*–S249A and *cdc13*–S255A strains. However, the *cdc13*–Q250A and *cdc13*–Q256A mutations had little to no effect on telomere length (Figure 5), in contrast to the more substantial telomere shortening observed in the *cdc13*–S249A and *cdc13*–S255A strains.

A potential caveat to this result is that binding of Tel1 (or Mec1) to an adjacent SQ motif in the proposed S/TQ cluster might be sufficient to phosphorylate either S255 or S249 in the *cdc13*–Q250A and *cdc13*–Q256A strains. Therefore, two additional mutant alleles of CDC13 were constructed, in which the glutamine residue was mutated to alanine at additional candidate phosphorylation motifs. As shown in Figure 5, a strain bearing mutations in four glutamine residues encompassing all four SQ motifs in the recruitment domain of Cdc13 (*cdc13*–Q226A, Q250A, Q256A, Q307A, referred

FIGURE 4.—Cdc13 phospho-isoform(s) are unaffected by mutations in the recruitment domain or loss of Tel1 function. (A) Extracts were prepared from a protease-deficient yeast strain expressing either Cdc13-(myc)₁₈ or Cdc13-S249A, S255A-(myc)₁₈ treated/mock-treated with λ-phosphatase, resolved on 6% PAGE and examined for the presence of the Cdc13-(myc)₁₈ protein by anti-myc Western. (B) Extracts prepared from isogenic *TEL1* and *tel1-Δ* strains expressing Cdc13-(myc)₁₈ were analyzed as in A.

to as *cdc13-4Q*→*4A*) exhibited a telomere length just slightly shorter than that of wild type. Finally, to eliminate the possibility that *Tel1* or *Mec1* were accessing the telomerase recruitment domain by binding a consensus SQ or TQ site anywhere in the Cdc13 protein, an allele of *CDC13* was constructed in which all 11 SQ and TQ consensus sites were mutated to SA or TA, respectively. The telomere length of the resulting *cdc13-11Q*→*11A* strain again was only slightly reduced, relative to wild type (Figure 5).

Thus, telomere homeostasis is essentially unaffected in a strain expressing a variant of Cdc13 that does not contain even a single consensus phosphorylation site for the *Tel1*/*Mec1* kinases. The validity of this experiment depends on the specificity of *Tel1* and/or *Mec1* kinases for substrates that contain glutamine in the +1 position. This has been addressed by a quantitative phosphoproteomics study, which identified 30 phosphorylation sites, in 28 different proteins, which were direct targets of *Mec1* and/or *Tel1* (see Table 2; SMOLKA *et al.* 2007). The majority of these sites (25 events) occurred at an S/TQ motif, with an additional 3 sites with a conservative replacement for glutamine (glutamic acid or asparagine) at the +1 position. The two remaining phosphorylation events occurred at sites with either serine or isoleucine at +1. This unbiased study demonstrates that *Mec1* and *Tel1* have a very high specificity for S/TQ motifs. Furthermore, on the basis of a survey of the published literature, we have not identified a single example of *Mec1*- or *Tel1*-dependent phosphorylation of serine or threonine residues with alanine in the +1 position. Taken together, the results shown in Figures 3, 4, and 5 argue against the proposal that *in vivo* telomere length is regulated by *Tel1*-dependent (or *Mec1*-dependent) phosphorylation of the proposed S/TQ cluster in the Cdc13 recruitment domain.

Additional residues in the Cdc13 recruitment domain are required for interaction with Est1: As a further step in our analysis of the Cdc13 recruitment domain, we introduced alanine mutations into conserved residues in the region spanning amino acids 235 to 254, on the basis of the alignment shown in Figure 1A. Strains expressing alanine mutations in three conserved residues—P235, F237, and F253—resulted in a sub-

stantial reduction in telomere length, whereas mutations in other residues in this domain had little (N254) or no (S225, N251, and S306) effect (Figure 6A). Mutations in residues P235, F237, and F253 also exhibited impaired ability to interact with *Est1* as assessed by a two-hybrid test (Figure 6B), indicating that the telomere length defect exhibited by mutations in these three residues is due to a reduced interaction with the *Est1* telomerase subunit. In contrast, a Cdc13-N254A two-hybrid fusion protein retained interaction with *Est1* in this assay (data not shown), consistent with the slight *in vivo* defect displayed by the *cdc13-N254A* strain.

We also examined the effects of several double mutant combinations in this region. The *cdc13-S249A*, *S255A* mutant strain exhibited a more severe telomere replication defect than the *cdc13-S255A* single mutant strain, displaying a senescence phenotype that was comparable to that of the *cdc13-2* strain (Figure S1 and Figure S5). This additive effect was not specific to these two SQ motifs, however, as a second double mutant strain (*cdc13-F253A*, *S255A*) also exhibited a more severe telomere replication defect than the *cdc13-S255A* single mutant strain, which was again comparable to that of the *cdc13-2* strain. Mutations in the two other proposed SQ motifs in this domain (*cdc13-S225A* and *cdc13-S306A*) did not enhance the severity of the phenotype displayed by a *cdc13-S255A* mutation (Figure S5), consistent with the wild-type telomere length of strains bearing either of these two mutations (Figure 6A). We conclude that the S₂₂₅Q and S₃₀₆Q sites are completely dispensable for telomere length regulation at native chromosome termini.

Epistasis analysis between the *TEL1* pathway and the telomerase recruitment pathway: Epistasis analysis has previously demonstrated that a *tel1-Δ* mutation partially attenuates the senescence phenotype of a *tlc1-Δ* strain (RITCHIE *et al.* 1999; ABDALLAH *et al.* 2009). As pointed out by PETES and colleagues (RITCHIE *et al.* 1999), this result is not easily explained by a model in which the primary role of *Tel1* at telomeres is to target a regulatory component of the telomerase pathway. We revisited this epistasis result, by comparing how the senescence progression of a *tlc1-Δ* strain was influenced by the presence of *tel1-Δ* or *tel2-1* mutations *vs.* mutations in the re-

FIGURE 5.—Telomere length of a *cdc13-11Q*→*11A* strain, which lacks all consensus Tel1/Mec1 phosphorylation sites. Telomere length analysis of the indicated mutations in *CDC13* transformed into the *cdc13-Δ/pCDC13 URA3* shuffle strain (YVL3006); strains were propagated for ~50 generations following eviction of the covering *CDC13* plasmid, prior to preparing genomic DNA for analysis of telomere length. The bracket indicates the 1.2 to 1.5 kb Y'-containing telomeric restriction fragment, which corresponds to approximately two-thirds of the telomeres in this strain background.

recruitment domain of *CDC13*. Senescence was assayed through a genotype-blind assessment of the growth characteristics of multiple isolates of a *tlc1-Δ* strain, compared to multiple isolates of a *tlc1-Δ* strain bearing an additional mutation. This protocol relies on a large number of samples to deal with the inherent variability of the senescence phenotype of telomerase-defective strains, with genotypes determined only at the conclusion of the experiment (see MATERIALS AND METHODS and Figure S6 for more details). We have previously validated this approach by complementary techniques, establishing it as a semiquantitative approach (RIZKI and LUNDBLAD 2001).

As expected, on the basis of prior observations (RITCHIE *et al.* 1999; ABDALLAH *et al.* 2009), the senescence progression of the *tel1-Δ* *tlc1-Δ* strain was slightly attenuated relative to that of a *TEL1* *tlc1-Δ* strain (Figure 7A), with a difference between the two genotypes that was statistically significant. Similarly, the *tel2-1*

FIGURE 6.—Additional residues in the Cdc13 recruitment domain mediate the interaction with Est1. (A) Telomere length analysis of the indicated mutations in *CDC13*, analyzed as described in Figure 5; white dots indicate a restriction fragment polymorphism that frequently occurs in these strains in the 1.2–1.5 kb Y'-containing telomeric restriction fragment (indicated by a bracket). (B) Two-hybrid analysis of the interaction between Cdc13–R635A (wild-type or mutant versions of pVL3125) and Est1 (pVL693), as monitored by the ability to activate the *GAL1-HIS3* reporter gene; Figure S2 shows that these same Gal4DBD–Cdc13 proteins are not impaired, as assessed by their ability to interact with Stn1 in a two-hybrid assay.

mutation, which functions in the same pathway as *Tel1* for DNA damage signaling (ANDERSON *et al.* 2008), also partially alleviated the senescence phenotype resulting from loss of telomerase (Figure 7B). In contrast, there was no difference between the senescence phenotypes of *tlc1-Δ* and *tlc1-Δ cdc13-2* strains, consistent with previous observations (NUGENT *et al.* 1996). Similarly, neither *cdc13-S255A* nor the *cdc13-S249A, S255A* double mutation altered the senescence progression of a *tlc1-Δ* strain (Figure 7, C, D, and E). These observations confirm that the recruitment function of *Cdc13*, as

FIGURE 7.—(A–E) Epistasis analysis of *tlc1-Δ* strains, with or without mutations in the Tel1/Tel2 pathway or the Cdc13 recruitment domain. For each epistasis test, a heterozygous diploid strain was freshly dissected and *tlc1-Δ* isolates were streaked for three successive streak-outs (corresponding to ~25, ~50, and ~75 generations of growth); numbers in parentheses represent the number of isolates of each genotype analyzed. Streak-outs were incubated for 48 hr at 30°, followed by a genotype-blind assessment of the growth phenotype. Each histogram displays the percentage of isolates (*x*-axis) that fell into five phenotypic categories (*y*-axis), ranging from a scale of 1 (severe senescence) to 5 (comparable to wild-type growth); see Figure S6 for examples of each of these five categories, as well as an evaluation of the validity of the analysis used to evaluate the statistical significance. The *cdc13*–*S249A*, *S255A* epistasis test was only performed once; all other epistasis tests were performed at least twice, with similar results.

defined by mutations in E252 and S255, acts in the telomerase pathway. In contrast, even though *tel1-Δ* and *tel2-1* mutations confer short telomeres (LUSTIG and PETES 1986), defects in these two genes can partially rescue the growth defect of a telomerase null strain, indicating that the Tel1/Tel2-mediated pathway performs an activity that is distinct from regulating telomerase function *in vivo*.

DISCUSSION

Association between *Est1* and the recruitment domain of *Cdc13* is essential for telomerase to be recruited to its site of action (EVANS and LUNDBLAD 1999; PENNOCK *et al.* 2001; BIANCHI *et al.* 2004; CHAN *et al.* 2008). This recruitment step has been proposed to be

regulated by phosphorylation of a proposed S/TQ cluster in the recruitment domain of *Cdc13* (TSENG *et al.* 2006). However, three observations presented in this study argue against this conclusion. First, the pattern of *Cdc13* phosphatase-sensitive isoforms is not altered by loss of *Tel1* or *Mec1* function or by mutations introduced into two conserved serines (S249 and S255) in this domain. Second, an interaction between *Cdc13* and *Est1*, as monitored by a two-hybrid assay, is dependent on S255 but *Tel1* independent. Finally, a derivative of *Cdc13*, *cdc13*–(S/TQ)_{II}→(S/TA)_{II}, in which every potential consensus phosphorylation site for *Tel1* or *Mec1* has been eliminated, retains nearly wild-type telomere length. Collectively, these results are not consistent with *Tel1*-mediated phosphorylation of the *Cdc13* S/TQ cluster regulating its interaction with *Est1*.

Unequivocal evidence that a protein is phosphorylated usually relies either on immunological detection with phosphoepitope-specific antibodies, or direct monitoring of protein phosphorylation status by phosphopeptide mapping, or mass spectrometry analysis. Initial indications that *Cdc13* might be regulated by phosphorylation came from a quantitative phosphoproteomics study, which identified two candidate phosphorylation sites on *Cdc13* at S₃₀₆Q and T₃₀₈P (SMOLKA *et al.* 2007). An *in vivo* role for phosphorylation of each of these two sites has been subsequently established by directed studies. The T₃₀₈P motif of *Cdc13* is targeted by the Cdk kinase, and defects in this phosphorylation step confer an ~75-bp decline in telomere length (LI *et al.* 2009), whereas the S₃₀₆Q site is phosphorylated by *Mec1* in response to DNA damage, with subsequent consequences for access of *Cdc13* to double-strand breaks (ZHANG and DUROCHER 2010). In both cases, the development of phospho-specific antibodies directed at these two sites has provided direct evidence that *Cdc13* is phosphorylated *in vivo* at T₃₀₈P and S₃₀₆Q. In contrast, a phospho-specific antibody directed at the phosphorylated version of S₂₅₅Q was not capable of recognizing S255-specific phosphorylation of *Cdc13* expressed in yeast (Figure S4 and data not shown). Therefore, the biochemical evidence that S₂₄₉Q and S₂₅₅Q are targets of *Tell* and/or *Mec1* rests solely on *in vitro* kinase assays monitoring the phosphorylation status of GST fusions to 75- to 90-amino-acid portions of *Cdc13* in *Tell* or *Mec1* immunoprecipitates (TSENG *et al.* 2006); however, such *in vitro* approaches can be notoriously promiscuous with regard to target specificity and thus often do not reflect the status of *in vivo* phosphorylation.

It is worth emphasizing that we only examined the *cdc13*–(S/TQ)₁₁→(S/TA)₁₁ strain for telomere length, leading to the conclusion that phosphorylation of *Cdc13* by *Tell* (or *Mec1*) is not required for telomere length regulation. However, this result does not exclude the possibility that S/TQ residues of *Cdc13* (in addition to the S₃₀₆Q site mentioned above) may be phosphorylated by *Tel* or *Mec1* for other regulatory purposes, such as in response to DNA damage.

An alternative model for the role of *Tell* in telomere homeostasis: Multiple studies have shown that association of telomerase with telomeres is highly regulated. *Est1* and *Est2* association peaks during late S phase, when telomerase elongation occurs (MARCAND *et al.* 2000), and this association is partially *Tell* dependent (SABOURIN *et al.* 2007). *Est1* and *Est2*, as well as *Tell*, also exhibit an enhanced interaction with a telomere which has been experimentally shortened (BIANCHI and SHORE 2007; SABOURIN *et al.* 2007). In contrast, telomeric association of *Cdc13* is independent of both *Tell* and telomere length, which suggests that the preferential recognition of short telomeres by telomerase occurs after *Cdc13* binding. As a result of these observations, a

prevailing model in the field proposes that *Tell* binding to telomeres marks them for elongation through phosphorylation of the recruitment domain of *Cdc13*. Enhanced association of *Tell* with shorter telomeres, with subsequent phosphorylation of *Cdc13* at this population of telomeres, provides a satisfying explanation for the ability of telomerase to preferentially target shorter telomeres.

However, if *Tell* does not phosphorylate the telomerase recruitment domain of *Cdc13*, as we argue in this current study, this indicates that the molecular mechanism for *Tell*-mediated length regulation has not yet been solved. We would like to repropose an alternative model for *Tell* at telomeres, which is one variant of a general model originally put forth by the PETES group (see RITCHIE *et al.* 1999; RITCHIE and PETES 2000 for a more detailed discussion). This model stems from the need to provide an explanation for why loss of *Tell* attenuates the senescence of a telomerase-defective strain. As pointed out by RITCHIE *et al.* 1999, this result is inconsistent with the idea that the sole function of *Tell* at telomeres is to target a component of the telomerase pathway. When considering alternative models, we considered the numerous parallels between double-strand breaks and telomeres. In particular, there is a modest delay in the accumulation of single-stranded DNA resection products at experimentally induced double-strand breaks in a *tel1*–Δ strain (MANTIERO *et al.* 2007). We propose that resection of chromosome ends is similarly slightly impaired in the absence of *Tell*. Such a molecular defect at telomeres provides a straightforward explanation for the deferred senescence phenotype of a telomerase-defective strain that lacks *Tell*, since reduced resection would decrease the telomere shortening rate (although telomeres still would ultimately become critically short, in the absence of telomerase).

In a telomerase-proficient strain that lacks *Tell*, we suggest that the shorter G-rich overhang, which would be the consequence of reduced resection, provides a suboptimal substrate for elongation by telomerase. Thus, in this model, the telomere length defect observed in *tel1*–Δ cells is an indirect consequence of a resection defect, rather than a direct consequence of altered regulation of telomerase. Although there is no direct evidence in support of this second aspect of the model, it is nevertheless consistent with the reduction in the overall frequency of elongation events by telomerase in *tel1*–Δ cells (ARNERIC and LINGNER 2007) as well as the reduced telomeric association of *Est1* and *Est2* in a *tel1*–Δ strain. BLACKBURN and colleagues have also previously proposed that the requirement for *Tell*/*Mec1* at telomeres could be bypassed by enhancing the extent of the single-stranded overhang to increase telomerase accessibility (CHAN *et al.* 2001).

One observation that has to be reconciled with the above proposal is that the interaction of *Cdc13* with

chromosome termini during late S phase is not altered in the absence of *Tell1*. Virtually every model about *Cdc13* assumes that *Cdc13* interacts solely with the single-stranded G-rich overhang present at the extreme termini of chromosomes. However, biochemical studies have established that *Cdc13* does not require a free 3' DNA terminus for binding and can bind equally well to internal tracts of G-rich telomeric DNA (NUGENT *et al.* 1996). *A priori*, *Cdc13* could potentially interact with the G-rich single-stranded region that is exposed when the replication fork moves through the duplex region of telomeric repeats; if so, this would be consistent with the association of *Cdc13* with chromosome termini that is observed during late S phase. In fact, the method used to monitor *Cdc13* telomeric association (chromatin immunoprecipitation) cannot distinguish between binding to the terminal G-rich overhang *vs.* binding to single-stranded regions exposed by the replication fork. Therefore, the *Tell1*-independent association of *Cdc13* with telomeres does not argue for or against a change in the length of the G-rich overhang in *tel1-Δ* strains. The recent demonstration that the *Cdc13*-associated *Stn1* and *Ten1* proteins exhibit notable similarities with subunits of the RPA complex (GAO *et al.* 2007; GELINAS *et al.* 2009; SUN *et al.* 2009; PASCHINI *et al.* 2010) also lends credence to the idea that a telomere-dedicated RPA complex could be a part of the replication machinery as it passes through the duplex telomeric tract.

This alternative model for *Tell1* also extends to the *Mre11* complex (MRX), which acts in the same pathway for telomere length as *Tell1* (NUGENT *et al.* 1998; RITCHIE and PETES 2000). Like *Tell1*, association of *Est1* and *Est2* with telomeres is reduced in *mrx-Δ* strains (TAKATA *et al.* 2005; GOUDSOUZIAN *et al.* 2006), and mutations in the MRX complex partially alleviate the consequences of a *tlc1-Δ* mutation (T. B. TORO and V. LUNDBLAD, data not shown). MRX-defective cells also exhibit altered resection at both double-strand breaks and native telomeres (LARRIVEE *et al.* 2004; TAKATA *et al.* 2005; MANTIERO *et al.* 2007). Thus, our model argues that the primary defect at telomeres—reduced resection of the telomeric C strand—occurs when either *Tell1* or the MRX complex is defective, analogous to the defect that is observed at double-strand breaks when either of these two activities is defective.

There is, however, one *Tell1*-related phenomenon that is not addressed by the above model, which is that the extent of telomerase-mediated elongation at extremely short telomeres (<125 bp) is attenuated in the absence of *Tell1* (CHANG *et al.* 2007). Specifically, the highly processive telomerase activity that is observed at these extremely short telomeres becomes nonprocessive in a *tel1-Δ* strain. CHANG *et al.* (2007) have argued that this particular subpopulation cannot arise as the result of normal telomere erosion and instead may be the consequence of replication fork collapse. If so, such

aberrant termini may be recognized and processed, at least initially, through regulatory events that are different than the events that occur at chromosome termini that have been fully replicated.

What regulates preferential association of telomerase with short telomeres? The current model in the field presumably answers this question: association of *Tell1* with short telomeres, and subsequent phosphorylation of the telomerase recruitment domain of *Cdc13*, ensure that telomerase is directed to the shortest telomeres. However, telomerase can still distinguish short from long native telomeres in the absence of *Tell1* (ARNERIC and LINGNER 2007). Furthermore, the S-phase peak of association of *Est1* and *Est2* is diminished, but not eliminated, in the absence of *Tell1* (SABOURIN *et al.* 2007). Thus, although the overall frequency of telomerase elongation events is reduced in a *tel1-Δ* strain (ARNERIC and LINGNER 2007), the basic regulatory mechanisms that direct telomerase to telomeres in S phase, and preferentially to short telomeres, remain intact. Combined with the results that we report here, the basis for preferential elongation of a particular subpopulation of telomeres remains an unresolved question.

The recruitment domain of *Cdc13* has a single regulatory function: In addition to residues immediately adjacent to the location of the *cdc13-2* allele, this study also examined the effect of mutations across a conserved ~25-amino-acid domain, including residue P235, which had previously been implicated in telomere length regulation. Analysis of the *cdc13-4* mutation (P235S) led to the proposal that this mutation defined a separate function for *Cdc13*, distinct from the recruitment function impaired by the *cdc13-2* mutation (MEIER *et al.* 2001). If P235 is in fact required for a different biochemical activity, this predicts that the *Cdc13*-P235S mutant protein should still retain the ability to interact with *Est1*. However, as shown in Figure 5, the *cdc13-4* mutation (P235S), as well as a mutation in an adjacent residue (F237), failed to interact with *Est1* as assessed by two hybrid. Furthermore, a *cdc13-2,4* strain exhibited a telomere replication defect that was indistinguishable from that of a *cdc13-2* strain (BUCKLEY 2005). We conclude that the region around P235 is required for telomerase recruitment, rather than for a separate function. More generally, our analysis of residues adjacent to the location of the original *cdc13-2* mutation defines a minimal ~25-amino-acid region that is required for interaction with the *Est1* telomerase subunit.

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GENETICS

Supporting Information

<http://www.genetics.org/cgi/content/full/genetics.110.122044/DC1>

Telomerase Recruitment in *Saccharomyces cerevisiae* Is Not Dependent on Tel1-Mediated Phosphorylation of Cdc13

**Hua Gao, Tasha B. Toro, Margherita Paschini, Bari Braunstein-Ballew,
Rachel B. Cervantes and Victoria Lundblad**

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FIGURE S1.—(A) Senescence phenotype of representative samples of the indicated strains, at ~25 generations (1X) up to ~125 generations (5X), analyzed as described in Figure 1. (B) Telomere length analysis of a *cdc13-S255A* strain, after propagation for ~125 generations prior to preparing genomic DNA. The bracket corresponds to the Y'-containing heterogeneous telomeric restriction fragment, which is 1.2 - 1.5 kb in a wild type strain. Unit length sub-telomeric Y' elements (present in two sizes, referred to as Y'-long and Y'-short; black arrowheads) undergo recombination-dependent amplification in extensively propagated telomerase-defective strains (LUNDBLAD and BLACKBURN 1993); Y' amplification in the *cdc13-S255A* strain is indicated by the white dots.

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FIGURE S2.—(A) Comparison of Est1 and Cdc13 two-hybrid constructs used in this study *vs.* those used previously by QI and ZAKIAN (2000). QI and ZAKIAN observed a weak interaction between the C-terminal 200 aa of Est1 and Cdc13, which was not affected by the *cdc13-2* mutation (as expected, as the C-terminal 200 aa domain of Est1 did not encompass the *est1-60* mutation, which defines the region of Est1 required for interaction with Cdc13; PENNOCK *et al.* 2000). (B) Two-hybrid analysis of the interaction between Stn1 (pVL859) and the Cdc13-R635A constructs examined in Figure 6B. (C) Isogenic derivatives of the PJ69-4A strain used for two hybrid analysis (JAMES *et al.* 1996), grown on rich media following loss of a covering *TEL1* plasmid, which was maintained in the strain during introduction of the *mec1-Δ* disruption and subsequently lost by propagation on 5-FOA-containing media.

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FIGURE S3.—Comparison of the pattern of phospho-isoforms of the wild type Cdc13-(myc)₁₈ protein with (A) Cdc13-T308A-(myc)₁₈, which eliminates a previously characterized Cdk-dependent phosphorylation site in Cdc13 (Li *et al.* 2009) or (B) in the absence of Tel1 and/or Mec1 function.

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A **B**

FIGURE S4.—A phosphorylated GST-Cdc13_{aa234-265} protein is recognized by an antibody raised against the phosphorylated version of S255. (A) Wild type or mutant versions of a GST-Cdc13_{aa234-265} fusion protein were expressed in *E. coli*, affinity purified and incubated with DNA-PK (Promega) in the presence of [γ -³²P] at 30° for 10 min. Reaction products were separated by 10% SDS-PAGE and phosphorylated products were visualized by autoradiograph, which demonstrates that both S249 and S255 can be phosphorylated *in vitro* by DNA-PK. (B) GST-Cdc13_{aa234-265} and GST-Cdc13_{aa234-265}-S255A were affinity purified and treated either with DNA-PK, as described in (A), or in mock reactions, and examined by western analysis with a polyclonal rabbit antibody raised against a peptide spanning aa 242 to 258 which was phosphorylated at S255 (Bethyl Antibodies). The western demonstrates that the phosphorylated version of S255, but not the phosphorylated version of S249, is recognized by this antibody.

FIGURE S5.—A *cdc13-Δ* / p *CDC13 URA3* shuffle strain (YVL3006) was transformed with plasmids expressing *cdc13-S255A* or the indicated double mutant combinations. The covering *CDC13 URA3* plasmid was evicted by propagation on 5-FOA-containing media; subsequent streak-outs were on rich (YPAD) media. Each pair of genotypes were compared in side-by-side streak-outs; a total of 8 samples were examined for each, with a representative set of examples shown. The three timepoints shown here correspond to ~50, ~75 and ~100 generations of growth. Plates were photographed after incubation for three days at 30°.

B

Method	p-value		
	1X	2X	3X
A	p = 0.002	p = 0.0003	p < 0.0001
B	p = 0.002	p = 0.002	p < 0.0001
C	p = 0.002	p = 0.0003	p < 0.0001
D	p = 0.005	p = 0.0003	p < 0.0001
E	p = 0.005	p = 0.003	p < 0.0001

FIGURE S6.—The statistical analysis shown in Figure 7 is based on the unvalidated assumption that there is a linear relationship with regard to the change in growth rate between each of the five categories; i.e. the assumption that the growth scores are equally weighted (method A, above). However, if there is a non-linear relationship between the different growth categories, this could potentially invalidate the statistical analysis. To test this, growth scores for several representative data sets were weighted according to five different scales, as shown in part (A), and the p-values for each were re-calculated. The table in (B) shows the data in Figure 7A evaluated by these five methods, which demonstrates that changes in the relative weightings of the growth rates for the five growth categories does not change the overall result.