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Cdc13 Delivers Separate Complexes to the Telomere for End Protection and Replication

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Summary

In *Saccharomyces cerevisiae*, the telomere binding protein Cdc13 mediates telomere replication by recruiting telomerase, and also performs an essential function in chromosome end protection. We show here that delivery of the Stn1 protein to the telomere, by fusing the DNA binding domain of Cdc13 (DBD_{CDC13}) to Stn1, is sufficient to rescue the lethality of a *cdc13* null strain and, hence, provide end protection. Telomere replication is still defective in this strain, but can be restored by delivering telomerase to the telomere as a DBD_{CDC13}-telomerase fusion. These results establish Stn1 as the primary effector of chromosome end protection, whereas the principal function of Cdc13 is to provide a loading platform to recruit complexes that provide end protection and telomere replication.

Introduction

Telomeres, the ends of linear chromosomes, need to solve two fundamental problems in order to ensure stable chromosome transmission and long term viability of the cell. First, the cell must be able to distinguish natural chromosome termini from newly broken DNA ends (reviewed in Lundblad, 2000). This is achieved primarily through the action of telomere-bound proteins that protect chromosome termini from the mechanisms that repair double-strand breaks, such as nonhomologous end joining. In budding and fission yeasts, defects in end protection can lead to unregulated degradation of one strand of the telomere, as well as chromosomal end-to-end fusions (Garvik et al., 1995; Nakamura et al., 1998; Diede and Gottschling, 1999; McEachern et al., 2000). Similar consequences for telomere dysfunction are also observed in mammalian cells. For example, titration of the duplex telomere DNA binding protein TRF2 from human telomeres results in end-to-end fusions and subsequent growth arrest or apoptosis (van Steensel et al., 1998; Karlseder et al., 1999). Second, the cell must employ mechanisms to ensure the complete replication of chromosome ends, which otherwise shorten with each cell division (reviewed in Evans and Lundblad, 2000). The primary means of telomere replication in most organisms relies on the enzyme telomerase, which elongates the 3′ terminus of the G-rich strand of the telomere using an internal RNA subunit as the template to dictate the sequence added to chromosome ends (Greider and Blackburn, 1989; Singer and Gottschling, 1994; Feng et al., 1995). In both yeast and human cells, a telomerase deficiency leads to a progressive decline in telomere length that inhibits the proliferative capacity of the cell and heralds replicative senescence (Lundblad and Szostak, 1989; Bodnar et al., 1998).

In *S. cerevisiae*, four genes encoding components of the telomerase enzyme have been identified that, when mutated, result in an inability to replicate the telomere. *EST2* and *TLC1* encode the reverse transcriptase catalytic protein subunit and the templating RNA, respectively (Singer and Gottschling, 1994; Counter et al., 1997; Lingner et al., 1997a), whereas *EST1* and *EST3* encode additional subunits of the telomerase holoenzyme (Lundblad and Szostak, 1989; Morris and Lundblad, 1997; Hughes et al., 2000a). The single strand telomere binding protein Cdc13 is also required for telomere replication, as both a positive and negative regulator of telomerase. A role for Cdc13 in positive regulation was uncovered with the identification of a novel missense mutation, *cdc13-2*, which confers the same in vivo defects as a telomerase-deficient strain, but has no discernible effects on telomerase enzyme activity (Nugent et al., 1996; Lingner et al., 1997b). These observations, as well as others, indicate that Cdc13, while bound to chromosome termini, recruits telomerase as the result of an interaction between Cdc13 and a subunit of the enzyme (Nugent et al., 1996; Evans and Lundblad, 1999; Qi and Zakian, 2000; this work). Alternatively, telomerase can be delivered to the telomere by fusing the high affinity DNA binding domain of Cdc13 (DBD_{CDC13}) to a telomerase subunit, which results in substantial telomere elongation and also bypasses the requirement for the telomerase recruitment activity of Cdc13 (Evans and Lundblad, 1999; Hughes et al., 2000a). Cdc13 has a separate distinct role in negative regulation of telomere replication that occurs subsequent to telomerase recruitment, and which Cdc13 performs in conjunction with the Stn1 protein (Grandin et al., 1997, 2000; Chandra et al., 2001). Stn1 and Cdc13 interact through an association that is abolished by the *cdc13-2* mutation, the same mutation that eliminates the positive regulatory interaction between Cdc13 and telomerase. This has led to the proposal that Cdc13 performs its two regulatory steps as a result of successive binding of telomerase and the negative regulator Stn1 to overlapping binding sites on Cdc13 (Chandra et al., 2001).

Cdc13 and Stn1 also both have at least one additional essential function at the telomere. During normal telomere replication, transient extended single-strand G-rich extensions are observed at yeast telomeres during late S phase (Wellinger et al., 1993). These single-strand extensions (dubbed "G tails"), which are detected even in cells that lack telomerase, have been proposed to arise as the consequence of cell-cycle-regulated degradation of the complementary C-rich strand (Wellinger et al., 1996). Since unregulated degradation should be lethal, an essential role for telomeric end protection would be to limit the extent of this C-strand-specific degradation. In fact, loss of *CDC13* function is accompanied by immediate and extensive strand-specific degradation of the

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telomeric C strand, resulting in a *RAD9*-dependent cell cycle arrest (Weinert and Hartwell, 1993; Garvik et al., 1995; Diede and Gottschling, 1999). Similarly, alterations in *STN1* function also result in a *RAD9*-dependent arrest and alterations in telomeric end structure (Grandin et al., 1997). These phenotypes suggest that the essential function of both of these proteins is to help prevent unregulated degradation of chromosome ends. Binding of Cdc13 to telomeric DNA is presumably required for this process (Hughes et al., 2000b), but whether Cdc13 makes additional biochemical contributions to chromosome end protection has not been determined, and the role of Stn1 in preventing degradation has not been elucidated at all. Thus, the basic mechanism of chromosome end protection in yeast remains unknown.

Since the Cdc13 protein promotes telomere replication by recruiting a complex—the telomerase enzyme—to the telomere, we asked whether Cdc13 might similarly perform its essential function by delivering an end protection complex to the telomere. We demonstrate that the lethality of a *cdc13-*Δ null strain can be rescued by delivering Stn1 to the telomere, as a fusion of the Stn1 protein to the minimal DNA binding domain of Cdc13 (DBD_{CDC13}-Stn1). Since the DBD_{CDC13} alone is incapable of providing end protection, this shows that an Stn1-containing complex is responsible for a critical process in yeast telomere biology—that of protection of the ends of chromosomes. However, the *cdc13-*Δ/*DBD_{CDC13}-STN1* strain still exhibits the diagnostic features (progressively shortened telomeres and eventual senescence) of a telomerase-defective strain, indicating that the DBD_{CDC13}-Stn1 fusion protein has restored end protection but not telomere replication. Telomere replication can be reestablished by delivery of telomerase to the telomere as a DBD_{CDC13}-telomerase fusion; however, because this mode of telomerase access is greatly enhanced (Evans and Lundblad, 1999), a *cdc13-*Δ strain bearing both the DBD_{CDC13}-Stn1 and DBD_{CDC13}-telomerase fusions exhibits overelongated telomeres. We addressed this by using an additional means of restoring telomere replication that instead utilized the normal route for telomerase recruitment. This final experiment relied on the identification of an ~15 kDa telomerase recruitment domain of Cdc13: placement of this minimal recruitment domain at chromosome termini, in combination with the DBD_{CDC13}-Stn1 fusion, is sufficient to reconstitute telomeres in a *cdc13-*Δ strain.

Therefore, telomere replication and protection of chromosome termini can be achieved via the delivery of separate complexes to the telomere. This work also identifies Stn1, rather than Cdc13, as the primary mediator of telomeric end protection, and demonstrates that the principal role for Cdc13 at the telomere is to bind telomeric DNA and recruit a succession of complexes. Since mammalian telomeres are also dependent on both replication and end protection processes, similar recruitment mechanisms may be required in human cells as well.

Results

Delivery of *STN1* to the Telomere Is Sufficient to Confer the Essential Function of *CDC13*

Several lines of evidence, including work presented below, demonstrate that Cdc13 promotes telomere replication by recruiting telomerase to the ends of chromosomes. By analogy, Cdc13 might similarly mediate its essential function by recruiting an end protection complex to telomeres. Since loss of *STN1* function has the same detrimental consequence for telomere metabolism as loss of *CDC13* function, the role of Cdc13 in end protection may be solely to mediate access of Stn1 (and possibly other Stn1-associated proteins) to the telomere. To test this hypothesis, we made a construct designed to deliver Stn1 to the telomere in the absence of *CDC13* function, by fusing the DNA binding domain of Cdc13 (DBD_{CDC13}) to the N terminus of Stn1. This fusion protein, expressed by the *CDC13* promoter on a low copy plasmid, complemented an *stn1-*Δ strain (data not shown), indicating that the presence of the DBD_{CDC13} had not altered *STN1* activity.

Remarkably, the *DBD_{CDC13}-STN1* fusion was able to efficiently rescue the lethality of a *cdc13-*Δ strain (Figure 1A). The ability to alleviate the requirement for *CDC13* function was a property specific to the Stn1 protein, since *cdc13-*Δ lethality was not rescued by either the DBD_{CDC13} alone or by fusions of the DBD_{CDC13} to four other proteins that also function in telomere metabolism (Est1-DBD_{CDC13}, Est3-DBD_{CDC13}, DBD_{CDC13}-Pol α, and DBD_{CDC13}-Ku) (Figure 1A and data not shown); each of these four fusions was able to complement the respective *est1-*Δ, *est3-*Δ, *pol1-12^{ts}* or *yku80-*Δ mutation (Evans and Lundblad, 1999; Hughes et al., 2000a; and data not shown). Therefore, the ability of the DBD_{CDC13}-Stn1 protein fusion to alleviate the requirement for *CDC13* function was not simply the consequence of delivery of any large protein to the chromosome terminus. Rescue of *cdc13-*Δ lethality was also not due to an increase in the steady-state levels of Stn1 protein, since high-level expression of the wild-type Stn1 protein by the constitutive high-level *ADH1* promoter was not sufficient to confer the growth conferred by even the low copy *DBD_{CDC13}-STN1* fusion construct (Figure 1A and data not shown). Furthermore, a mutant $DBD_{CDC13\text{-}6}$-Stn1 fusion protein, carrying the thermolabile DNA binding defective mutation *cdc13-6* (Hughes et al., 2000b), conferred viability to the *cdc13-*Δ strain at 23°C but not 36°C, indicating that the DNA binding property of the DBD_{CDC13} was required (data not shown). These results indicate that the essential function of *CDC13*, and hence cell viability, can be maintained if the Stn1 protein is delivered to the telomere by the high affinity DBD_{CDC13}. This suggests that the primary effector of chromosome end protection is Stn1, and that the role of Cdc13 in this process is to deliver Stn1 to the telomere.

The *cdc13-*Δ/*DBD_{CDC13}-STN1* Strain Has a Telomere Replication Defect

The viability of the *cdc13-*Δ/*DBD_{CDC13}-STN1* strain could be due to restoration of normal telomere function; alternatively, this genetic situation could create a novel solution to telomere maintenance. However, examination of telomere structure in the *cdc13-*Δ/*DBD_{CDC13}-STN1* strain revealed that telomeres looked relatively normal: there were no notable differences in the overall pattern of telomeric restriction fragments, relative to a wild-type strain (Figure 1B). This indicated that the ability to bypass lethality was not a consequence of striking rearrangements at telomeres or other obvious changes in genome structure, such as chromosome circularization.

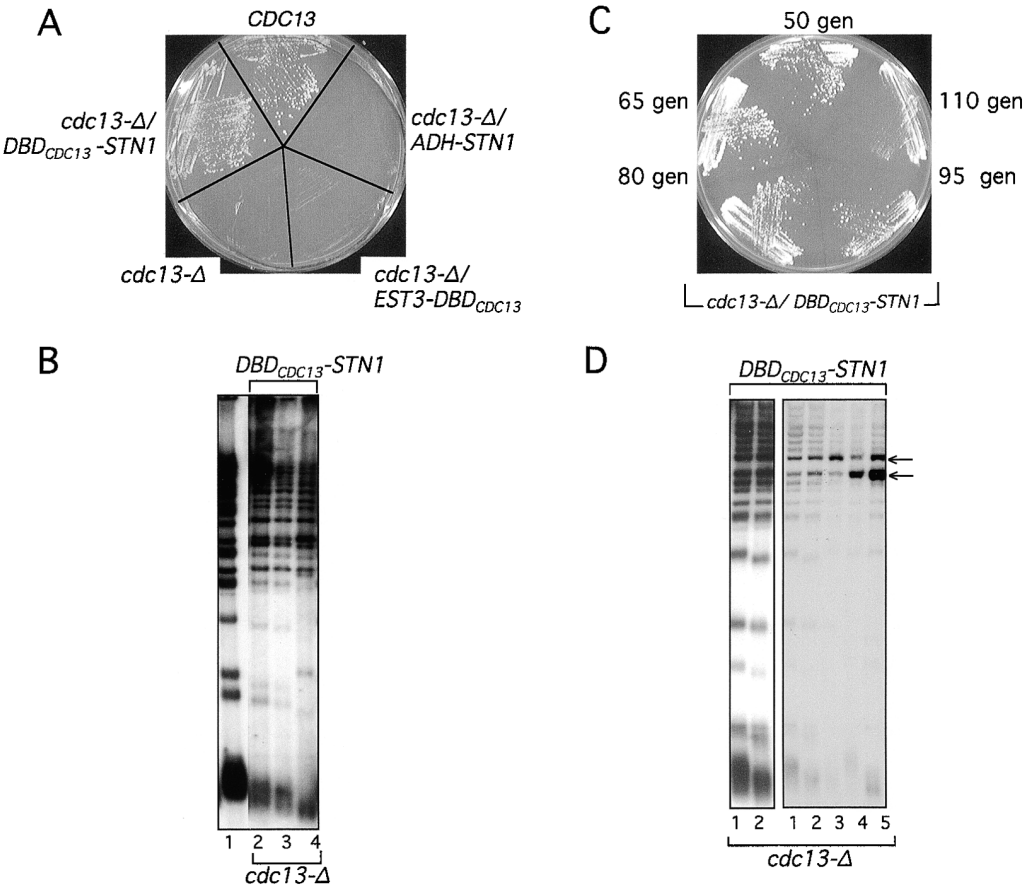

Figure 1. The *DBD$_{CDC13}$-STN1* Fusion Rescues the Essential Function of *CDC13*

(A) The *DBD$_{CDC13}$-STN1* fusion rescues the lethality of a *cdc13-Δ* null strain. Growth of *cdc13-Δ/CDC13* (pVL1086), *cdc13-Δ/ADH-STN1* (pVL1131), *cdc13-Δ/DBD$_{CDC13}$-STN1* (pVL1253), *cdc13-Δ/EST3-DBD$_{CDC13}$* (pVL1292) and *cdc13-Δ*/vector control (pRS425) strains. Plasmids were introduced into a haploid *cdc13-Δ*/pVL438 (*CEN URA3 CDC13*) strain; growth of the viable strains corresponds to ~25 generations after eviction of pVL438.

(B) A *cdc13-Δ/DBD$_{CDC13}$-STN1* strain exhibits progressive telomere shortening. A genomic Southern blot, probed with poly d(GT/CA), shows the telomere length of a *CDC13* strain (lane 1) or a *cdc13-Δ/DBD$_{CDC13}$-STN1* strain (lanes 2–4) propagated by serial culturing for ~30, 45, or 60 generations of growth, respectively, after loss of a covering *CDC13* plasmid.

(C) A *cdc13-Δ/DBD$_{CDC13}$-STN1* strain displays a senescence phenotype. The *cdc13-Δ/DBD$_{CDC13}$-STN1* strain was propagated by serial culturing, and an aliquot from each successive culture was streaked onto media selecting for the plasmid and incubated at 30°C for 48 hr. The strain used in this experiment was generated independently from that shown in (B).

(D) Prolonged propagation of the *cdc13-Δ/DBD$_{CDC13}$-STN1* strain produces type I survivors. A genomic Southern blot of a *cdc13-Δ/DBD$_{CDC13}$-STN1* strain, propagated by serially culturing, results in telomeric and subtelomeric rearrangements, indicative of a telomerase-independent mode of telomere maintenance. A darker exposure (left side) illustrates telomere shortening during early propagation (lanes 1 and 2, 30 and 45 generations of growth), and a lighter exposure (right side; lanes 1–5, 30 to 95 generations of growth) is shown to illustrate the amplification of sub-telomeric Y′ elements (indicated by the two arrows), which is a characteristic feature of one type of telomerase-defective survivors (Lundblad and Blackburn, 1993). In other experiments, rearrangements characteristic of the other class of survivors were observed (data not shown).

Although overall telomere structure in the *cdc13-Δ/DBD$_{CDC13}$-STN1* strain looked normal, telomere length was reduced (Figure 1B), suggesting that telomerase recruitment could not occur in the absence of the intact Cdc13 protein. Consistent with this proposal, additional telomere shortening was observed with continued propagation (Figure 1B; see also Figure 1D), resulting in eventual senescence (Figure 1C). Furthermore, after extended propagation of the *cdc13-Δ/DBD$_{CDC13}$-STN1* strain, healthy growing survivors were identified (Figure 1D) that exhibited rearrangements of telomeric and subtelomeric sequences that are characteristic of the survivors recovered from telomerase-defective strains (Lundblad and Blackburn, 1993). The growth defect of the *cdc13-Δ/DBD$_{CDC13}$-STN1* strain was further exaggerated by the introduction of a *rad52*$^-$ mutation (data not shown), consistent with previous demonstrations that the appearance of telomerase-defective survivors is blocked by a *rad52*$^-$ mutation (Lundblad and Blackburn, 1993; Lendvay et al., 1996). Therefore, the *cdc13-Δ/DBD$_{CDC13}$-STN1* strain exhibits an *est*$^-$ phenotype, based on all of the criteria that have been previously used to characterize telomerase-defective strains.

Delivery of Separate End Protection and Telomere Replication Complexes to the Telomere

We have previously shown that fusion of the DBD$_{CDC13}$ to either the Est1 or Est3 telomerase subunits results

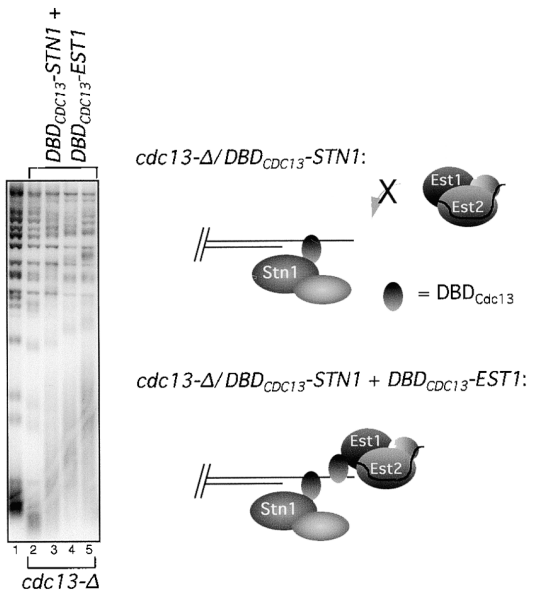

Figure 2. The Telomere Replication Defect of a *cdc13-Δ/DBD$_{CDC13}$-STN1* Strain Is Restored by the Introduction of a DBD$_{CDC13}$ -Telomerase Fusion Protein

A *cdc13-Δ*/pVL438 (*CEN URA3 CDC13*) strain was cotransformed with plasmids expressing the *DBD$_{CDC13}$-STN1* fusion (pVL1372) and the *EST1-DBD$_{CDC13}$* fusion (pVL1120), followed by eviction of the covering *CDC13* plasmid and serial propagation in liquid culture. Lane 1, a *CDC13* strain; lanes 2–5, a *cdc13-Δ/DBD$_{CDC13}$-STN1*+ *EST1-DBD$_{CDC13}$* strain propagated for ~30, 45, 60, 80, and 95 generations of growth following loss of the covering plasmid. The schematic figure presents an interpretation of the results in Figures 1 and 2: in the absence of intact Cdc13 protein, the minimal DBD$_{CDC13}$ can be used to deliver an Stn1-containing complex (hypothetically drawn as a two-subunit complex) and the telomerase holoenzyme to the chromosome terminus to restore telomere function. Whether multiple DBD$_{CDC13}$ -containing complexes can simultaneously bind to the same telomere is considered in the Discussion.

in substantial telomere elongation, indicating that the affinity of the enzyme for chromosome termini has been greatly increased (Evans and Lundblad, 1999; Hughes et al., 2000a). Furthermore, these DBD$_{CDC13}$-telomerase fusions are capable of bypassing the recruitment-defective *cdc13-2* mutation. If the *est*$^-$ phenotype of the *cdc13-Δ/DBD$_{CDC13}$-STN1* strain is due to an inability of telomerase to access the telomere, then this predicts that the telomere replication defect of the *cdc13-Δ/DBD$_{CDC13}$-STN1* strain should similarly be alleviated by the introduction of a DBD$_{CDC13}$ -telomerase fusion protein. As predicted, the introduction of the Est1-DBD$_{CDC13}$ fusion protein reversed the telomere shortening of the *cdc13-Δ/DBD$_{CDC13}$-STN1* strain; instead, a *cdc13-Δ* strain carrying both fusions exhibited substantial telomere elongation (Figure 2), comparable to that previously observed when an Est1-DBD$_{CDC13}$ fusion protein was present in a wild-type strain (Evans and Lundblad, 1999). In addition, this strain now exhibited normal growth, with no evidence of senescence (data not shown). These results show that the two functions of Cdc13 can be physically separated, by using the DBD$_{CDC13}$ to deliver two different complexes to the telomere (as presented schematically in Figure 2). Thus, in the absence of intact Cdc13 function, the DBD$_{CDC13}$-Stn1 fusion confers an essential protective function but cannot replicate the telomere (Figure 1), whereas DBD$_{CDC13}$-telomerase fusions promote telomere replication (Evans and Lundblad, 1999; Hughes et al., 2000a) but cannot provide the essential protective function (Figure 1A).

Reconstituting a Wild-type Telomere

The experiment depicted in Figure 2 demonstrates that telomere function and long-term viability can be restored even in the absence of the intact Cdc13 protein. However, this experiment relied on a DBD$_{CDC13}$-telomerase fusion as a means of restoring telomere replication, which resulted in overreplicated telomeres rather than wild-type telomere length, presumably due to greatly increased levels of telomerase recruitment. As an alternative approach, we asked whether we could restore normal levels of telomerase recruitment, and hence wild-type telomere length, using the *cdc13-Δ/DBD$_{CDC13}$-STN1* strain as a starting point. A schematic representation of our experimental plan is shown in Figure 3A. This experiment relies on the assumption that the telomerase recruitment function resides within a specific domain of the Cdc13 protein. To localize this proposed domain, we examined the properties of a set of protein fusions (termed DBD$_{CDC13}$-RD fusions, where "RD" stands for the telomerase recruitment domain) that linked the minimal DNA binding domain with the proposed telomerase recruitment region. Eleven nested regions of Cdc13, ranging from amino acids 190 to 340, were fused in frame at the C-terminal boundary of the DNA binding domain (note that this order is the opposite to that found in the native protein; see Figure 3A). To test for telomerase recruitment activity, each fusion was first transformed into the recruitment-defective *cdc13-2* strain. Three fusions (DBD$_{CDC13}$-RD$_{aa190–340}$, DBD$_{CDC13}$-RD$_{aa211–340}$ and DBD$_{CDC13}$-RD$_{aa211–331}$) rescued the senescence and telomere length defect of the *cdc13-2* strain, whereas the comparable mutant fusions, containing the *cdc13-2* missense mutation at amino acid 252 (denoted as DBD$_{CDC13}$-RD^{est}) were unable to complement *cdc13-2* (Figure 3B and data not shown).

If this 120 amino acid region is sufficient to recruit telomerase, then the DBD$_{CDC13}$-RD fusion should also rescue the telomere replication defect of the *cdc13-Δ* null mutant kept alive with the DBD$_{CDC13}$-Stn1 fusion protein. Figures 3C and 3D show that the DBD$_{CDC13}$-RD fusion was capable of rescuing the *est*$^-$ phenotype of the *cdc13-Δ/DBD$_{CDC13}$-STN1* strain. In a *cdc13-Δ* strain carrying both the DBD$_{CDC13}$-Stn1 and the DBD$_{CDC13}$-RD protein fusions, telomeres were stably maintained at a length close to that of wild type (Figure 3C), and as expected, the strain no longer displayed a senescence phenotype (Figure 3D). In contrast, a mutant DBD$_{CDC13}$-RD^{est} fusion protein, even in high copy, failed to rescue the telomere replication defect of the *cdc13-Δ/DBD$_{CDC13}$-STN1* strain (see Figure 4D and data not shown). These experiments indicate that a 120 amino acid domain is necessary and sufficient to provide the Cdc13 telomerase recruitment activity. Furthermore, this work shows that a wild-type telomere can be fully reconstituted in a strain that lacks the intact Cdc13 protein, when alternative means are used to deliver end protection and telomerase recruitment activities to the telomere.

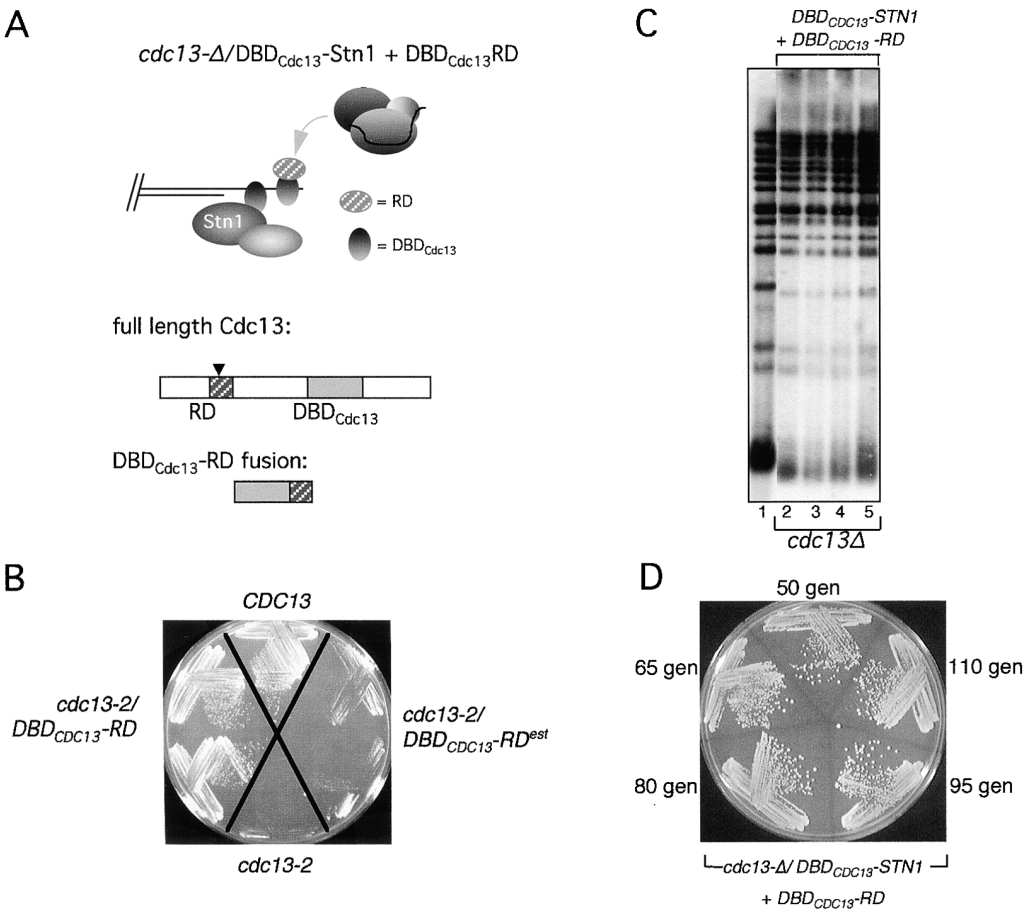

Figure 3. Reconstituting a Wild-type Telomere in the Absence of Intact Cdc13 Protein

(A) Experimental plan for restoring the normal pathway for telomerase recruitment in a *cdc13-*Δ strain kept alive by the DBD_{CDC13}-Stn1 fusion protein. In this strategy, telomerase access to the telomere is proposed to occur as the result of an interaction between the holoenzyme and a minimal telomerase recruitment domain of Cdc13, which is present at the telomere as a DBD_{CDC13}-RD fusion protein. The construction of the DBD_{CDC13}-RD fusion is also shown: the proposed recruitment domain of Cdc13p is indicated by the striped box, with the residue altered in the recruitment-defective *cdc13-2* mutation (E252K) denoted by the black arrowhead, and the DNA binding domain of Cdc13p is represented by the shaded box. Note that the order of the RD and DBD_{CDC13} domains in the DBD_{CDC13}-RD fusion is the opposite of that in the native protein.

(B) A DBD_{CDC13}-RD fusion protein complements the senescence phenotype of the *cdc13-2* mutant. Growth after ~75 generations of a *cdc13-2* strain harboring plasmids expressing the wild-type Cdc13 protein (pVL1086), a vector control (pRS425), the DBD_{CDC13}-$RD_{aa190–340}$ fusion (pVL1269) or the DBD_{CDC13}-$RD^{est}_{aa190–340}$ version (pVL1368).

(C) A *DBD_{CDC13}-RD* fusion prevents progressive telomere shortening in the *cdc13-*Δ*/DBD_{CDC13}-STN1* strain. A *cdc13-*Δ/pVL438 (*CEN URA3 CDC13*) strain was cotransformed with a plasmid expressing the *DBD_{CDC13}-STN1* fusion (pVL1253) and the *DBD_{CDC13}-RD* fusion (present in single copy; pVL1383) and genomic DNA was prepared from serially propagated cultures. Lane 2 corresponds to ~30 generations of growth following loss of the covering *CDC13* plasmid, and each subsequent lane represents an additional ~12 generations of growth; lane 1, a *CDC13* strain.

(D) A *DBD_{CDC13}-RD* fusion prevents the senescence of a *cdc13-*Δ*/pDBD_{CDC13}-STN1* strain. A *cdc13-*Δ*/pDBD_{CDC13}-STN1* + *pDBD_{CDC13}-RD* (2 μ; pVL1384) strain was generated and propagated by serial culturing as described in Figure 1C; the approximate number of generations following loss of the covering *CDC13* plasmid is indicated.

The Recruitment Domain of Cdc13 Interacts with a Specific Site on Telomerase

Cdc13 is not tightly associated with telomerase, as assessed by immunoprecipitation experiments in which all components are expressed in single copy under native promoters (Evans and Lundblad, 1999; Hughes et al., 2000a). Therefore, if the telomerase recruitment domain of Cdc13 interacts with the enzyme directly, it is not a tight and/or stable association. However, increasing the levels of the wild-type Est1 protein partially suppresses the telomere replication defect of the *cdc13-2* mutant (Nugent et al., 1996), and recombinant versions of the Est1 and Cdc13 proteins can be coimmunoprecipitated when both are overexpressed (Qi and Zakian, 2000). This suggests that the telomerase-associated Est1 protein binds to a site on Cdc13 defined by the *cdc13-2* mutation, although these experiments do not rule out the possibility of an additional protein required to bridge this association.

To provide evidence for a direct interaction between these two proteins, we screened for mutation(s) in *EST1* that could specifically suppress the *cdc13-2* defect. A mutagenized library of an *EST1 CEN* plasmid was introduced into a *cdc13-2 rad52*$^-$ strain (see Experimental

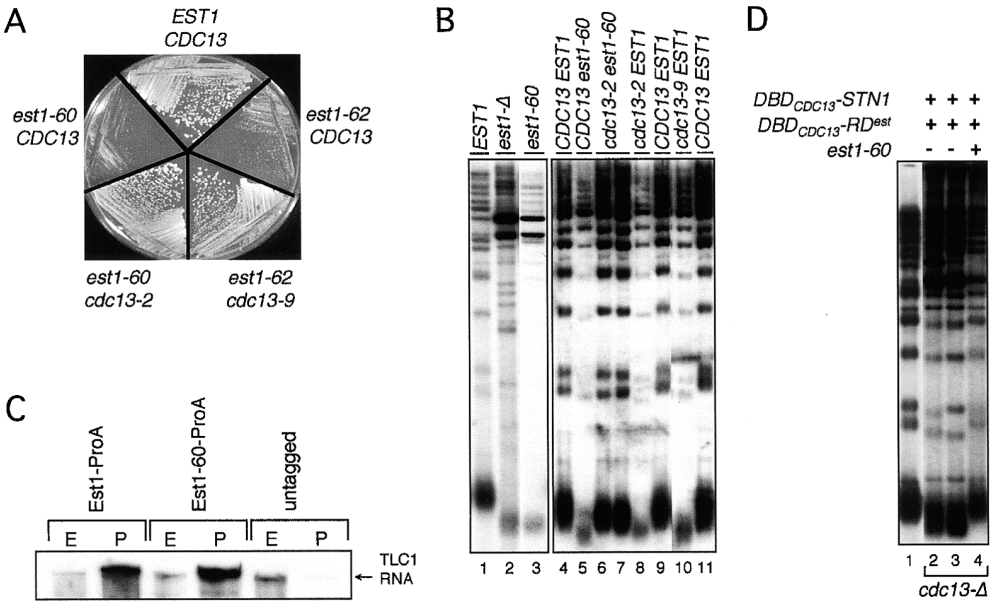

Figure 4. The Recruitment Domain of Cdc13 Interacts with a Specific Site on the Telomerase-Associated Est1 Protein

(A) The *est1-60* and *cdc13-2* mutations exhibit mutual suppression. A haploid *est1-*Δ *cdc13-*Δ/pVL438 (*CEN URA3 CDC13*) strain was cotransformed with plasmids expressing *CDC13* (pVL1084) and *EST1* (pVL198); *CDC13* (pVL1084) and *est1-62* (pVL1720); *cdc13-9* (pVL1726) and *est1-62* (pVL1720); *cdc13-2* (pVL690) and *est1-60* (pVL 1689); and *CDC13* (pVL1084) and *est1-60* (pVL1689). Transformants were subsequently propagated on plates containing 5-FOA to evict the covering *CDC13* plasmid. The *est1-60 CDC13* and *est1-62 CDC13* strains are shown at their maximum senescence point (80 to 100 generations after the loss of *EST1* function); the *est1-60 cdc13-2*, and *est1-62 cdc13-9* strains are shown after propagation for an additional 50 generations.

(B) Telomere length of *est1* and *cdc13* mutant strains. Lane 1, *est1-*Δ/pVL232 (*EST1*); lane 2, *est1-*Δ/YCplac33; lane 3, *est1-*Δ/pVL1372 (*est1-60*); genomic DNA was prepared from very senescent *est1-*Δ and *est1-60* strains, which exhibit telomeric and subtelomeric rearrangements that are characteristic of late senescing *est*$^-$ mutants (Lundblad and Blackburn, 1993). Lanes 4–7 show the telomere length of the reciprocally suppressed *cdc13-2 est1-60* double mutant strain: lane 4, *CDC13 EST1*; lane 5, *CDC13 est1-60*; and lanes 6 and 7, *cdc13-2 est1-60*. Lane 8, *cdc13-2 EST1*; lanes 9 and 11, *CDC13 EST1*; and lane 10, *cdc13-9 EST1*.

(C) The Est1-60 protein retains association with telomerase. Extracts were prepared from an *EST1* strain expressing Est1-ProA (containing a C-terminal protein A tag; pVL1375), Est1-60-ProA (pVL1569) or untagged Est1 (pVL232); the Est1 protein was immunoprecipitated and Northern blotting was performed to detect the telomerase TLC1 RNA subunit. Crude extract lanes (E) represent 3% of the input into the immunoprecipitation fraction (P). The efficiency of immunoprecipitation, as quantitated by PhosphorImager analysis, was 23% and 16% for Est1-ProA and Est1-60-ProA, respectively; recovery with the untagged protein was <1%.

(D) The *est1-60* mutation suppresses the recruitment defect of the DBD_{CDC13}-RD^{est} fusion protein. A *cdc13-*Δ/DBD_{CDC13}-*STN1* + *pDBD*$_{CDC13}$-RD^{est} (2 μ; pVL1386) strain that had been grown for ~30 generations in the absence of *CDC13* was transformed with either a plasmid containing the *est1-60* mutation (pVL1372; lane 4) or a vector control (pRS416; lanes 2–3); lane 1, a *CDC13* strain. DNA was prepared from cultures grown for ~30 generations following introduction of the relevant plasmid.

Procedures for details). Two alleles, *est1-60* and *est1-61*, were recovered that suppressed the replication defect of the *cdc13-2* mutation. The *est1-61* allele displayed no impairment of *EST1* function when transformed into an *est1-*Δ null mutant strain (data not shown). We presume that the ability of this allele to suppress *cdc13-2* was the consequence of an increase in either the level or activity of the Est1-61 protein, and this mutant allele is not considered further in this report.

In contrast, the *est1-60* allele exhibited a severe telomere replication defect that resulted in critically short telomeres and a senescence phenotype, comparable to that of an *est1* null strain (Figures 4A and 4B). However, a *cdc13-2 est1-60* double mutant strain displayed healthy growth (Figure 4A), with telomere length restored to nearly wild type (Figure 4B). Therefore, these two mutations reciprocally suppress each other, consistent with the idea that physical contact between these two proteins has been restored. These two mutations are both recessive (Lendvay et al. 1996, and data not shown), and suppression is allele-specific (data not shown), thereby fulfilling genetic criteria expected for mutations that restore protein–protein interaction (i.e., see Sandrock et al., 1997). Therefore, we propose that the *est1-60* defect is due to a mutation in a subunit of telomerase that decreases the ability of the enzyme to be recruited to the telomere, in parallel with our proposal that the *cdc13-2* mutation alters the ability of *CDC13* to recruit telomerase. This predicts that the Est1-60 mutant protein should still retain association with the telomerase holoenzyme complex, which was confirmed by the demonstration that the Est1-60 mutant protein immunoprecipitated the *TLC1* RNA subunit at a level comparable to that of the wild-type Est1 protein (Figure 4C).

Sequencing of the *est1-60* mutation identified a single mutational change at residue 444, resulting in a Lys → Glu change. Intriguingly, the *cdc13-2* allele had previously been shown to be the consequence of a Glu → Lys missense mutation (Nugent et al., 1996). Thus, the reciprocal suppression between these two recessive mutations is due to a charge swap, which further supports the premise that the original contact site between

two interacting proteins has been restored. This result also suggests that Est1 and Cdc13 may interact via electrostatic forces between two charged protein surfaces. This is supported by additional mutational analysis that introduced an Arg-Asp pair of mutations at these same two residues. Either of two single mutant strains, bearing either a *cdc13-9* (Glu → Arg at residue 252) mutation or an *est1-62* (Lys → Asp at reside 444) mutation, have a telomere replication defect (Figures 4A and 4B). Telomere replication is restored in the double mutant strain, *cdc13-9 est1-62* (Figure 4A and data not shown), once again demonstrating reciprocal suppression between a mutation in *CDC13* and a mutation in *EST1*. In contrast, strains that pair either two basic residues, or two acidic residues, at amino acid 444 of Est1 and amino acid 252 of Cdc13 (such as Glu/Glu, Lys/Lys, Lys/Arg, or Asp/Glu) exhibited defective telomere replication (Figures 4A and 4B and data not shown). Thus, when a pair of oppositely charged amino acids is present at these two positions, the interaction between Est1 and Cdc13 is retained, but a pair of residues with the same charge destroys the interaction.

This set of experiments also provided us with an additional set of reagents to probe the function of the minimal Cdc13 telomerase recruitment domain. If this 120 amino acid domain is restoring telomere replication through the same process as that employed by the intact Cdc13 protein, then the DBD_{CDC13}-RD fusion protein should display the same genetic interaction with mutant and wild-type alleles of *EST1*. Introducing the *cdc13-2* mutation into the DBD_{CDC13}-RD fusion protein, to generate the DBD_{CDC13}-RD^{est} fusion, abolished the ability to rescue the telomere replication defect of the *cdc13-Δ/DBD_{CDC13}-STN1* strain (Figure 4D and data not shown). Strikingly, this recruitment-defective DBD_{CDC13}-RD^{est} fusion could be completely suppressed by the *est1-60* mutation: telomeres were maintained at wild-type length in a *cdc13-Δ/DBD_{CDC13}-STN1* + *DBD_{CDC13}-RD^{est}* strain that also contained the *est1-60* mutation (Figure 4D; compare lanes 1 and 4). Thus, this small 15 kDa domain of Cdc13 is capable of mimicking the normal process of telomerase recruitment.

Discussion

Yeast Chromosome End Protection Requires Delivery of Stn1 to the Telomere

Although it is clear that Cdc13 is essential for maintaining the integrity of chromosome termini in yeast, the mechanism by which Cdc13 promotes end protection has not previously been elucidated. The work presented here shows that this essential function of *CDC13* can be provided by delivering Stn1 to the telomere. Neither the DBD_{CDC13} itself, nor fusions of other proteins to the DBD_{CDC13}, exhibit the ability to rescue the lethality of a *cdc13-Δ* strain, indicating that this is not a general consequence of placing any large protein at the telomere. Thus, Stn1 (and possibly Stn1-associated factors) appears to be the primary effector of telomeric end protection, providing a specific activity that prevents degradation of chromosome ends.

However, although the Stn1-DBD_{CDC13} protein confers end protection, the *cdc13-Δ/STN1-DBD_{CDC13}* strain exhibits the classic phenotypes of a telomerase-deficient strain. Restoration of telomere replication requires either of two additional fusions that reestablish telomerase access even in the absence of the intact Cdc13 protein. Collectively, these experiments show that the telomere replication and essential functions of Cdc13, previously proposed to be functionally distinct activities based on genetic analysis (Nugent et al., 1996), can be physically separated.

A Model for Cdc13 Function at the Telomere

Loss of *CDC13* function is accompanied by immediate and extensive strand-specific degradation of the telomeric C strand (Garvik et al., 1995; Diede and Gottschling, 1999), suggesting that Cdc13, while bound to the end of the chromosome, protects chromosome termini from unregulated degradation. This degradative activity is also proposed to play a role during normal telomere replication (Wellinger et al., 1996), by exposing a single strand region and thereby providing a substrate for binding by Cdc13. We therefore propose that the primary role of Cdc13 in telomere function is to bind the single-strand extensions present at chromosome termini, through its high- affinity single-strand telomere DNA binding domain, and to provide a protein platform for recruitment of other protein complexes (see Figure 5). These complexes—an Stn1-containing end protection complex and the telomerase holoenzyme—then provide the enzymatic and/or other functions that are necessary in order to both protect and replicate telomeres. Whether these complexes bind simultaneously to the same chromosome terminus (possibly delivered by the same Cdc13 molecule, as implied in Figure 5), or at temporally distinct times during the cell cycle, is not addressed by these studies. However, we have previously reported that the minimum binding site for Cdc13 is 11 nucleotides, and that successive Cdc13 proteins could bind to oligonucleotides of sufficient length (Hughes et al., 2000b). Since the G tail present at yeast telomeres is ≥30 nucleotides during S phase (Wellinger et al., 1993), it is possible that multimeric complexes may be similarly capable of forming on a single telomeric overhang, potentially performing separate end protection and replication functions.

Analysis of a Recruitment Domain of Cdc13

This model proposes a molecular view of how Cdc13 acts at the telomere: it is a protein that consists of a single strand DNA binding module that acts in conjunction with a series of recruitment domains. In support of this model, we have identified a 15 kDa telomerase recruitment domain of Cdc13, as well as demonstrating that Est1 is the direct binding partner for Cdc13-mediated telomerase recruitment.

Several prior observations had implicated the Est1 protein as the likely candidate for the Cdc13-interacting telomerase subunit. Fusion of Cdc13 directly to the catalytic core of telomerase allows continuous propagation and telomere maintenance in the complete absence of the Est1 protein, showing that a primary function of Est1, like Cdc13, is to facilitate enzyme recruitment (Evans and Lundblad, 1999). Furthermore, increasing the levels of wild-type Est1 protein partially suppresses the telo-

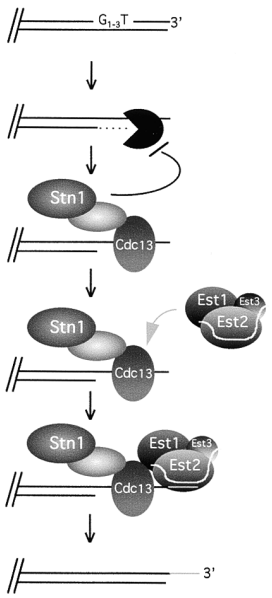

Figure 5. Model for the Role of Cdc13 in Telomere Replication and End Protection

The single-stranded G strand observed late in S phase, generated by a telomerase-independent mechanism (Wellinger et al., 1996), provides a substrate for binding by Cdc13. The processing activity that removes C strand sequences has not been identified and is shown hypothetically as a 5′ to 3′ exonuclease. We propose that binding of Cdc13 to the telomere is necessary for subsequent recruitment of two complexes that protect and replicate the telomere. The Stn1-containing end protection complex would inhibit further removal of C strand sequences, thereby protecting the telomere, followed by recruitment of telomerase by Cdc13, which elongates the G strand of the telomere. C-strand fill-in synthesis completes the process; whether Cdc13 remains bound to the telomere at this stage is not known and therefore not indicated. The negative regulatory function of Cdc13 and Stn1, which is not depicted in this figure, apparently requires a direct interaction between the two proteins (Grandin et al., 1997; Chandra et al., 2001); whether Cdc13 and Stn1 similarly execute their essential function through a direct interaction, or through an intervening additional protein (as hypothetically depicted here), is unknown.

mere replication defect of the *cdc13-2* mutant (Nugent et al., 1996), and recombinant versions of the Est1 and Cdc13 proteins can be observed in a complex when both are overexpressed (Qi and Zakian, 2000). However, these experiments did not establish whether the telomere-bound Cdc13 protein and the telomerase-associated Est1 protein bind each other directly to mediate telomerase recruitment, or whether another protein is required to bridge the proposed association. This issue is addressed in this work with the demonstration that a pair of oppositely charged residues at amino acid 252 of Cdc13 and amino acid 444 of Est1 dictates whether a cell is competent for telomere replication. These data argue that Est1 and Cdc13 interact directly via electrostatic forces between two charged protein surfaces in order to promote telomerase recruitment. It will be of interest to determine whether a similar interaction is required for mammalian telomerase recruitment.

This work has also led to the identification of an ~15 kDa domain in Cdc13 that is necessary and sufficient to provide the telomerase recruitment function. The activity of this domain is abolished by mutating residue 252 (corresponding to the *cdc13*-mutation in the intact protein), but restored by the presence of the suppressing *est1-60* mutation, indicating that all of the information needed by Cdc13 to respond to Est1 and mediate recruitment of the telomerase complex resides within this 120 amino acid region. By analogy, our model proposes that an additional region of Cdc13 is responsible for recruiting the proposed end protection complex; experiments are in progress to attempt to identify this second hypothesized recruitment domain.

Cdc13 and Stn1: Dual Players at the Telomere

Several studies have shown that Stn1 and Cdc13 perform an additional activity, as negative regulators of telomere length, which is distinct from the role of these two proteins in chromosome end protection (Grandin et al., 1997, 2000; Qi and Zakian, 2000; Chandra et al., 2001). Certain mutations in both *STN1* and *CDC13* disrupt normal telomere length regulation, resulting in telomeres that are elongated by more than 1000 bp (Grandin et al., 1997, 2000; Chandra et al., 2001). Since mutant alleles of DNA polymerase α give rise to similar phenotypes (Carson and Hartwell, 1985; Adams and Holm, 1996) and Cdc13 can be detected in a complex with DNA pol α (Qi and Zakian, 2000), this implicates Cdc13 as a regulator of synthesis of the C-rich strand of the telomere. We have proposed that the positive and negative regulatory roles of Cdc13 reflect two distinct steps in telomere replication: Cdc13 first recruits telomerase to the telomere and subsequently limits extension of the G strand by telomerase in response to C strand replication (Chandra et al., 2001). This second negative regulatory step is proposed to be mediated by a direct interaction between Cdc13 and Stn1 (Grandin et al., 1997, 2000; Chandra et al., 2001). Therefore, Cdc13 and Stn1 collaborate to perform two functionally distinct (and potentially temporally distinct) activities at the telomere: regulation of length homeostasis and protection of chromosome termini.

Potential Parallels to Mammalian Telomere End Protection

In human cells, chromosome end protection relies on the action of the telomere binding protein TRF2. Titration of TRF2 from telomeres leads to unprotected termini and consequent end-to-end chromosome fusions, indicating that TRF2 is an essential component of mammalian end protection (van Steensel et al., 1998). However, unlike Cdc13, TRF2 is bound to the duplex region of the telomere, rather than to the single-strand terminus. Furthermore, not only do Cdc13 and TRF2 differ in their substrate specificity, but loss of either protein leads to different molecular consequences for chromosome termini. Whereas a *CDC13* defect results in resection of the C strand of the telomere, loss of TRF2 from telomeres leads to loss of the 3′ G-rich single-strand overhang (van Steensel et al., 1998). This could suggest that yeast and human telomeres, when left exposed, are subject to different degradative processes. Alternatively, both strands of the telomere may be susceptible to degradation, in both yeast and human cells. If so, this would imply that there are mechanisms to inhibit 3′ to 5′ degra-

dation of the G strand as well as mechanisms to inhibit 5′ to 3′ degradation of the C strand; only one protective activity has been identified in each system.

Another proposed component of human telomere end protection is a structure formed by the terminal duplex tract called a t loop, where the 3′ terminus of the G strand invades the duplex region of the telomere (Griffith et al., 1999). This elegant structure has been proposed to provide an architectural solution to chromosome end protection, and might also contribute to regulation of telomere length by sequestering the terminus from telomerase. In vitro, TRF2 promotes t loop formation, and localizes to the junction where the 3′ overhang invades the duplex telomeric tract (Griffith et al., 1999). Although no mammalian equivalent to Cdc13 has yet been reported, the substrate specificity of Cdc13 is consistent with a role in t loop formation and/or stabilization (Nugent et al., 1996). Therefore, it is possible that a complex interplay of factors at both human and yeast telomeres will be needed in order to maintain the integrity of chromosome termini.

Experimental Procedures

Yeast Strains and Plasmids

All haploid strains used in this study were derived from a set of isogenic diploid strains descended from YPH275 (described in Lundblad and Szostak, 1989), by introduction of the relevant disruptions or the *cdc13-2* missense mutation by one-step gene disruption or allele replacement techniques. A haploid *cdc13-Δ* strain kept alive by a covering *CDC13* plasmid (pVL438; *CEN URA3 CDC13*), used for experiments throughout this paper, was derived from DVL162 (*MATa/α CDC13/cdc13Δ::LYS2 ura3-52/ura3-52 ade2-101/ade2-101 trp1Δ-1/trp1Δ-1 his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1*/CF-*SUP11-TRP1*/pVL 438). Haploid *cdc13-2*, *est1-Δ* or *est1-Δ cdc13-2* strains, *cdc13-2 rad52-Δ*, or *est1-Δ cdc13-Δ*/pVL438 strains were each freshly dissected as needed from DVL154 (*MAT* a/α *cdc13-2/CDC13 est1-Δ::HIS3/EST1 ura3-52/ura3-52 ade2-101/ade2-101 lys2-801/lys2-801 leu2-Δ1/leu2-Δ1 trp1-Δ1/trp1-Δ1 his3-Δ200/his3-Δ200*), DVL157 (*MATa/α cdc13-2/CDC13 rad52-Δ::LYS2/RAD52 ura3-52/ura3-52 ade2-101/ade2-101 lys2-801/lys2-801 leu2-Δ1/leu2-Δ1 trp1-Δ1/trp1-Δ1 his3-Δ200/his3-Δ200*) or DVL336 (*MATa/α est1-Δ::HIS3/EST1 cdc13-Δ::LYS/CDC13 ura3-52/ura3-52 ade2-101/ade2-101 lys2-801/lys2-801 leu2-Δ1/leu2-Δ1 trp1-Δ1/trp1-Δ1 his3-Δ200/his3-Δ200*/pVL438), respectively.

The DBD_{CDC13}-*STN1* plasmids used in this study were constructed by fusing the full-length *STN1* ORF (obtained from pVL1009, containing a 5.7 kb genomic insert encompassing the *STN1* gene in YEp351), in frame to the C terminus of two different variants of the *CDC13* DNA binding domain, to generate pVL1134 (*CEN LEU2* $DBD_{CDC13aa1–20,451–694}$-*STN1*) and pVL1253 (2 μ *LEU2* $DBD_{CDC13aa1–20,497–694}$-*STN1*); the fusion protein was expressed by the *CDC13* promoter. Derivatives of pVL1134 were obtained by standard cloning techniques to yield pVL1136 (2 μ *LEU2* $DBD_{CDC13\ aa1–20,451–694}$-*STN1*) and pVL1732 (2 μ *TRP1* $DBD_{CDC13\ aa1–20,451–694}$-*STN1*). The DBD_{CDC13}-RD plasmids were constructed from pVL1253, by replacing the *STN1* coding sequence with in-frame fusions of regions of *CDC13* from amino acids 190 to 340 to generate pVL1269 (2 μ *LEU2* DBD_{CDC13}-$RD_{aa190–340}$), pVL1273 (2 μ *LEU2* DBD_{CDC13}-$RD_{aa211–340}$), and pVL1358 (2 μ *LEU2* DBD_{CDC13}-$RD_{aa211–331}$), as well as other DBD_{CDC13}-RD fusion proteins not described in this report. Derivatives of pVL1269 and pVL1273 (denoted as DBD_{CDC13}-RD^{est}) were constructed by introduction of the *cdc13-2* missense mutation, to generate pVL1368 (2 μ *LEU2* DBD_{CDC13}-$RD^{est}_{aa190–340}$) and pVL1369 (2 μ *LEU2* DBD_{CDC13}-$RD^{est}_{aa211–340}$), respectively. pVL1368 was subcloned into a pRS424 backbone to generate pVL1386 (2 μ *TRP* DBD_{CDC13}-$RD^{est}_{aa190–340}$). High- and low-copy versions of pVL1269 were obtained by transferring the construct to a pRS414 backbone, creating pVL1383 (*CEN TRP1* DBD_{CDC13}-$RD_{aa190–340}$), or a pRS424 backbone, yielding pVL1384 (2 μ *TRP1* DBD_{CDC13}-$RD_{aa190–340}$). Details concerning the construction of pVL1120 (Est1-DBD_{CDC13}) and pVL1292 (Est3-DBD_{CDC13}) have been published elsewhere (Evans and Lundblad, 1999; Hughes et al., 2000a). pVL438 (*CEN URA3 CDC13*) or pVL1086 (*CEN LEU2* $CDC13_{myc18}$) were used as wild-type *CDC13* controls; the presence of the myc18 tag at the C terminus of Cdc13 in pVL1086 has no effect on Cdc13 function (C. Nugent, personal communication).

Mutational Analysis of *EST1* and *CDC13*

pVL232 (*CEN URA3 EST1*, derived from YCplac33) was passaged through a mutagenic strain of *E. coli*, XL1-Red (Stratagene), and transformed into a freshly generated *cdc13-2 rad52-Δ* haploid strain. Large colonies were picked from the transformation plate and examined for healthy growth upon additional propagation. Two strains were isolated that showed plasmid-dependent complementation of the growth and telomere length defects of the *cdc13-2* mutant strain. Plasmids were recovered from these two yeast transformants and sequenced, yielding pVL1372 (*CEN URA3 est1-60*) and pVL1373 (*CEN URA3 est1-61*). For telomerase immunoprecipitations, the *est1-60* mutation was subcloned into pVL1375 (*CEN TRP1 EST1-ProA*) to generate pVL1569 (*CEN TRP1 est1-60-ProA*). Additional mutations in *EST1* and *CDC13*, to generate *est1-62* (K444D; pVL1720) and *cdc13-9* (E252R; pVL1726) were constructed by site-directed mutagenesis of pVL198 (*CEN TRP1 EST1*) and pVL1084 (*CEN LEU2 CDC13*), respectively.

Genetic Methods

Yeast strain construction and telomere length determination by Southern blot analysis were performed as previously described (Lendvay et al., 1996). A plasmid shuffle strategy was used to introduce plasmids and investigate their ability to complement lethality of a *cdc13-Δ* strain: a *cdc13-Δ*/pVL438 (*CEN URA3 CDC13*) haploid strain was transformed with the relevant plasmid(s), maintaining selection for all plasmids, and colonies were subsequently streaked onto media containing 5-FOA to evict the covering wild-type plasmid but retain the newly introduced plasmid(s). For assays of telomere length and senescence phenotypes, colonies from the 5-FOA plate were inoculated into 8 ml of appropriate media and grown to saturation, followed by dilution and regrowth. Investigation of double mutant *est1 cdc13* combinations was done by transforming the relevant plasmids into a freshly dissected haploid *est1-Δ cdc13-Δ*/pVL438 (*CEN URA3 CDC13*) strain, followed by evicting pVL438 by plating on 5-FOA-containing media.

Telomerase Immunoprecipitations

Extracts were prepared from the protease-defective strain AVL78, carrying pVL232 (encoding an untagged version of Est1), pVL1375 (encoding Est1-ProA), and pVL1569 (encoding Est1-60-ProA), as described previously (Hughes et al., 2000a). Approximately 6 mg of extract was incubated with 40 μl IgG Sepharose beads (IgG Sepharose Fast Flow, Amersham Pharmacia Biotech) for 2 hr at 4°C to immunoprecipitate the Protein A–tagged Est1 proteins. The beads were then collected by gentle centrifugation, and the IP pellet was washed three times in TMG plus 200 mM NaCl, 0.5% Tween-20 and RNasin, and one time in TMG plus 50 mM NaCl and RNasin. RNA was prepared by phenol-chloroform extraction, separated on a 7 M urea/4% polyacrylamide gel, transferred to Hybond-N nylon membrane, and hybridized with a probe to detect the telomerase RNA (TLC1).

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