Distinct roles for yeast Stn1 in telomere capping and telomerase inhibition

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The budding yeast Cdc13, Stn1 and Ten1 (CST) proteins are proposed to function as an RPA-like complex at telomeres that protects ('caps') chromosome ends and regulates their elongation by telomerase. We show that Stn1 has a critical function in both processes through the deployment of two separable domains. The N terminus of Stn1 interacts with Ten1 and carries out its essential capping function. The C terminus of Stn1 binds both Cdc13 and Pol12, and we present genetic data indicating that the Stn1–Cdc13 interaction is required to limit continuous telomerase action. Stn1 telomere association, similar to that of Cdc13, peaks during S phase. Significantly, the magnitude of Stn1 telomere binding is independent of telomere TG tract length, suggesting that the negative effect of Stn1 on telomerase action might be regulated by a modification of CST activity or structure in *cis* at individual telomeres. Genetic analysis suggests that the Tel1 kinase exerts an effect in parallel with the Stn1 C terminus to counteract its inhibition of telomerase. These data provide new insights into the coordination of telomere capping and telomerase regulation.

Introduction

Telomeres in the budding yeast *Saccharomyces cerevisiae*, and nearly all other eukaryotes examined to date, comprise short repeated sequences (TG_{1-3} in yeast and T_2AG_3 in metazoans) that serve as a platform for the binding of proteins required for their replication and protection from degradation or end joining (reviewed in Verdun and Karlseder, 2007). The TG-rich DNA strand of telomeres, which forms the 3′ chromosome terminus, exists as a short single-stranded (ss) extension whose length increases during S phase through a regulated process of 5′ end resection of the CA-rich strand (reviewed in Chakhparonian and Wellinger, 2003). This 3′ TG-rich overhang serves as a binding site for the Cdc13

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protein, first identified for its essential role in telomere protection, or 'capping' (Garvik *et al*, 1995). In the absence of Cdc13 function, telomeres undergo extensive degradation, leading to a checkpoint-dependent cell cycle arrest in G2/M (Weinert and Hartwell, 1988; Lydall and Weinert, 1995). This essential capping function of Cdc13 appears to be carried out together with two other proteins, Stn1 and Ten1, both of which interact with Cdc13, as temperature-sensitive (Ts) lethal mutations in either gene also lead to telomere uncapping and a G2/M cell cycle arrest (Grandin *et al*, 1997, 2000, 2001). It is thus presumed that Cdc13, Stn1 and Ten1 act together in a complex (CST) that caps telomere ends.

Genetic analysis of *CDC13* has revealed a complex role for Cdc13 protein in telomere maintenance. Cdc13 has a direct function in the recruitment of telomerase, through an interaction with the accessory factor Est1 (Evans and Lundblad, 1999; Pennock *et al*, 2001; Bianchi *et al*, 2004), that can be genetically separated from its capping function (Nugent *et al*, 1996). As such, cells carrying a *cdc13-2* mutation undergo progressive telomere shortening and senescence, but their telomeres are not initially uncapped. The capping function of Cdc13 instead appears to be carried out by Stn1 (presumably together with Ten1), because fusion of the Cdc13 DNA-binding domain to Stn1 is sufficient for capping (Pennock *et al*, 2001). In addition to its essential role as a positive effector of telomerase function, Cdc13 is also implicated in negative regulation of telomere elongation, as removal of a C-terminal domain of the protein leads to extensive telomere elongation (Chandra *et al*, 2001).

The precise role of Stn1 in telomere maintenance, though less clear than that of Cdc13, also appears to be complex. As mentioned above, genetic and hybrid protein studies clearly indicate a role for Stn1 in telomere capping, probably carried out through direct interactions with Cdc13. However, a recent study shows that Stn1, when overexpressed, can provide a capping function in the absence of Cdc13 (Petreaca *et al*, 2006). *STN1* mutants also display a telomere elongation phenotype (Grandin *et al*, 1997), and Stn1 protein has been proposed to negatively regulate telomerase action by competing with Est1 for binding to Cdc13 (Chandra *et al*, 2001). Interestingly, Stn1 also interacts with the Pol12 subunit of the Pol α–primase complex (Grossi *et al*, 2004), itself implicated in telomere length regulation and capping (Carson and Hartwell, 1985; Adams Martin *et al*, 2000; Qi and Zakian, 2000). Finally, studies in the related yeast *Kluyveromyces lactis* implicate Stn1 in the repression of recombination-based telomere tract elongation and rapid deletion (Iyer *et al*, 2005).

Here, we report the results of a series of experiments designed to dissect the role of Stn1 in both telomere length regulation and capping. We begin by defining regions and specific residues of Stn1 required for interactions with Ten1, Cdc13 and Pol12, which are then correlated with specific telomere functions. To address in molecular detail the role of Stn1 in telomere length regulation, we employ chromatin

immunoprecipitation (ChIP) of Stn1 itself, or of Cdc13 and Est1, in cells carrying *STN1* mutations. Finally, we use genetic epistasis analysis to explore the relationship between Stn1 and other regulators of the telomerase pathway, in particular the MRX (Mre11, Rad50 and Xrs2) complex and Tel1 (the yeast ATM homologue). Our results are discussed in terms of recent findings concerning the TG tract length-dependent regulation of telomerase action.

Results

Two functionally distinct interaction domains in Stn1

Two-hybrid analysis has shown previously that Stn1 interacts with both Cdc13 and Ten1 (Grandin *et al*, 1997, 2001; Chandra *et al*, 2001), two other proteins required for telomere capping, as well as with Pol12, the B subunit of the DNA polymerase α–DNA primase complex (Grossi *et al*, 2004). To determine whether Stn1 interacts with these three proteins through different or common regions, we generated two Stn1 hybrids with the Gal4 activation domain (GAD). GAD–stn1-Nt expresses the first 281 amino acids of Stn1 fused to GAD, whereas GAD–Stn1-Ct expresses a C-terminal Stn1 fragment (amino acids 282–495). These Stn1 hybrids were tested against two full-length Gal4 DNA-binding domain (GBD) fusions (GBD–Cdc13 and GBD–Ten1), and a fusion of LexA to an N-terminal domain of Pol12 (LexA–Pol12-Nt), in the appropriate two-hybrid reporter strains.

We found that GAD–stn1-Nt failed to interact with either GBD–Cdc13 or LexA–pol12-Nt, but displayed a strong interaction with GBD–Ten1, comparable to that of full-length GAD–Stn1 (Figure 1A). Conversely, GAD–stn1-Ct interacted with both GBD–Cdc13 and LexA–pol12-Nt to levels near that

of full-length GAD–Stn1, but barely at all with GBD–Ten1 (Figure 1A). These data suggest that the C-terminal part of Stn1 (amino acids 282–495) is largely responsible for the Stn1 interaction with both Cdc13 and Pol12, whereas an N-terminal domain (1–281) binds to Ten1. To determine whether the stn1-Ct interaction with Pol12 and Cdc13 can be distinguished genetically, we mutagenized *STN1* and used the two-hybrid assay to screen for mutations defective in the LexA–pol12-Nt interaction. This allowed us to identify one allele of *STN1* (*stn1-5*) that displays a severely reduced interaction with both LexA–pol12-Nt and GBD–Cdc13. Interestingly, a second allele (*stn1-20*) fails to interact with LexA–pol12-Nt, but interacts normally with GBD–Cdc13 (Figure 1A). The functional consequences of these mutations will be described below.

Essential capping function of the Stn1 N terminus

The specificity of interaction shown by the two different Stn1 domains in two-hybrid assays (Figure 1A) prompted us to determine whether the C and N termini of Stn1 execute different functions. To test this hypothesis, we expressed either GAD–stn1-Nt or GAD–stn1-Ct in a strain in which the endogenous copy of *STN1* was deleted (*stn1-Δ*). This strain was kept alive by a centromeric plasmid containing *STN1* and the *URA3* gene (YCpLac33-STN1). After selecting for loss of YCpLac33-STN1 by growing cells on plates containing 5-FOA, we observed (Figure 1B) that GAD–stn1-Nt was able to rescue the lethality of a genomic *stn1-Δ* mutation, indicating that the N terminus of Stn1 (amino acids 1–281) is sufficient to carry out its essential function. Notably, GAD–stn1-Ct was unable to complement the lethality of the *stn1-Δ* mutation (Figure 1B). These data suggest that the stn1
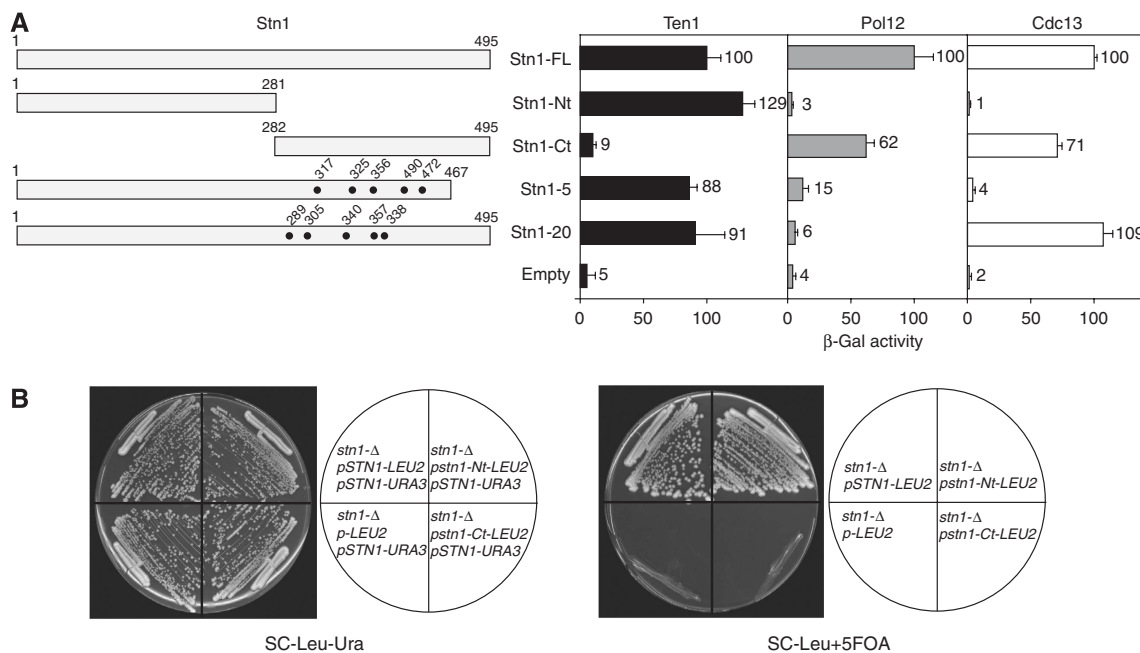

Figure 1 Identification of two interaction domains in Stn1. (**A**) Stn1 fusions to the Gal4 activation domain (GAD) are expressed from the pACT2 vector; Ten1 and Cdc13 fusions to the Gal4 DNA-binding domain are expressed from the pAS2 and pGBD vectors, respectively; a Pol12 fusion to LexA is expressed from the pLexA vector. All fusions are under the control of the *ADH1* promoter. Data are the average of two independent β-galactosidase measurements normalized to stn1-FL, arbitrarily set to 100. (**B**) GAD/stn1-Nt rescues the lethality of an *stn1*-null strain. In the diagrams at the right of each plate photograph, the plasmids present in each strain are indicated (all strains carry a full deletion of the genomic *STN1* gene). The names indicate both the *STN1* allele and the genetic marker present in each plasmid (STN1-URA3 = YCpLac33-STN1, STN1-LEU2 = pACT2-STN1, stn1-Nt-LEU2 = pGAD/stn1-Nt, stn1-Ct-LEU2 = pGAD/stn1-Ct, empty = pGAD-LEU2; see Table II for details).

interaction with Pol12 and Cdc13 is not necessary for cell viability, whereas its interaction with Ten1 is essential.

The observation that the Stn1 N terminus is indispensable for cell viability suggests that this portion of Stn1 carries out the essential function of telomere capping. To test this idea more directly, we generated a new *STN1* allele, *stn1-63*. Charbonneau and colleagues have shown that the *stn1-154* mutant displays a telomere elongation phenotype and a strong growth defect at 37°C, consistent with a Ts capping defect (Grandin *et al*, 2001). The *stn1-154* allele possesses three different point mutations, one of which (D99E) maps to a highly conserved portion of the N terminus, whereas the other two map to a variable portion very near the C terminus (Supplementary Figure S1A). To test whether the D99E substitution is responsible for the Ts phenotype of *stn1-154*, we generated an allele of *STN1* (*stn1-63*) containing only this point mutation in an otherwise wild-type *STN1* gene. We found that this mutant allele (either on a centromeric plasmid or overexpressed as a GAD fusion from a multicopy plasmid) conferred a severe growth defect at elevated temperatures in an *stn1-Δ* strain, comparable to that of the *stn1-154* allele (Figure 2A and data not shown). Furthermore, the *stn1-63* and *stn1-154* mutations display indistinguishable telomere elongation phenotypes at both 24°C and following a shift to 37°C (Supplementary Figure S1B).

To define more clearly the molecular defect caused by the *stn1-63* mutation, and the roles of the two different Stn1 domains in telomere maintenance, we quantified the extent of CA strand erosion at a single telomere in *stn1-Nt* and *stn1-63* strains, using an assay (QAOS; quantitative amplification of ss DNA) previously used to characterize the capping-defective Ts-lethal mutant *cdc13-1* (Booth *et al*, 2001). This method provides a measure of ss DNA in a subtelomeric region (in this case, approximately 8 and 14 kb internal to the

Chr. V-R telomere) and is thus a direct indicator of uncapping. The results in Figure 2B show that the *stn1-Nt* mutation had little or no influence on subtelomeric DNA structure, whereas the *stn1-63* Ts allele, at the non-permissive temperature, caused a significant increase in ss DNA at the two internal sites at Chr. V-R. The amount of ss DNA generated in the *stn1-63* mutant strain is less than that found in a *cdc13-1* mutant, consistent with the fact that the latter strain is considerably more thermo-sensitive (Figure 2A).

To determine whether the Stn1 N terminus is sufficient for normal telomeric DNA and chromatin structure, we performed additional analysis of the *stn1-Nt* strain. Using a sensitive hybridization assay to detect telomeric ss TG-repeat DNA (i.e. ss DNA at the telomere itself), we indeed found elevated levels in the *stn1-Nt* strain compared with wild type (Supplementary Figure S2A). This ss DNA is largely terminal as hybridization is effectively eliminated by treatment with exonuclease I. Despite this alteration in terminal DNA structure, we found that the *stn1-Nt* strain (as well as *stn1-63*) displayed normal levels of subtelomeric gene silencing, or telomere position effect (TPE, see Supplementary Figure S2B). As TPE relies upon the duplex TG-repeat binding factor Rap1 for its establishment (Kyrion *et al*, 1993; Moretti *et al*, 1994), this finding supports the notion that the telomere structure defect in the *stn1-Nt* strain is restricted to the very end of the telomere.

Stn1 C terminus is required for telomere length control

Genetic and two-hybrid data led Chandra *et al* (2001) to propose that the Stn1–Cdc13 interaction negatively regulates telomerase recruitment in *cis*. This model predicts that a reduction in association between Stn1 and Cdc13, such as that observed for stn1-Nt in a two-hybrid assay (Figure 1), should result in relaxed telomere length control. Indeed, we found that the expression of GAD–stn1-Nt in an *stn1-Δ* strain led to over-elongated telomeres (Figure 3A). Furthermore, *stn1-Δ* cells expressing GAD–stn1-5 (which fails to interact with Cdc13; see Figure 1A) display a telomere elongation phenotype similar to that of *stn1-Nt*, whereas the *stn1-20* allele, which interacts normally with Cdc13, but not with Pol12 (see Figure 1A), has little or no effect on telomere length (Figure 3A). Taken together, these data suggest that an interaction between the Stn1 C terminus and Cdc13 exerts a negative effect on telomere elongation.

The telomere over-elongation observed in the *stn1-Nt* strain could be the result of partial deregulation of telomerase, or of recombination-dependent effects. To test the latter possibility, we deleted *RAD52*, required for most homologous recombination events in yeast, in the *stn1-Nt* strain. This had no effect on telomere elongation caused by *stn1-Nt* (Figure 3B). In contrast, inactivation of telomerase in an *stn1-Nt* strain, by the introduction of the *est2D670A* mutation, caused telomere shortening and senescence (Figure 3C). These results indicate that telomere over-elongation in the *stn1-Nt* strain is due to an effect on telomerase.

Stn1 binds telomeres in a cell cycle-regulated but TG tract length-independent manner

Although the results described above suggest that Stn1 has a direct function in regulating both telomerase action and telomere capping, there has so far been no evidence reported to indicate if or when Stn1 is physically associated with
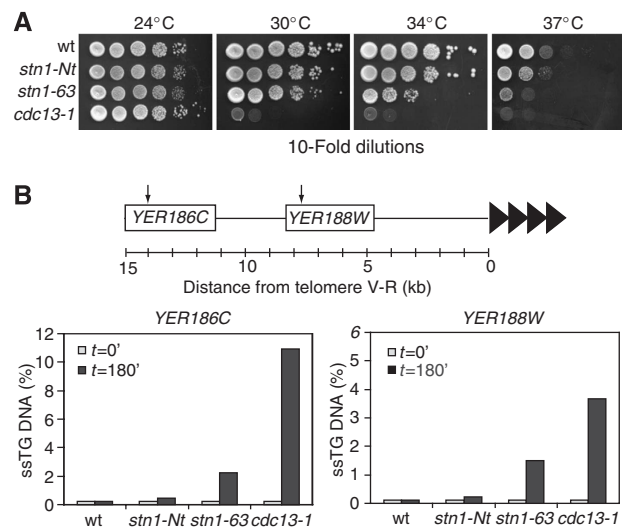

Figure 2 Temperature sensitivity and capping function of *stn1* mutant alleles. (**A**) Strains were grown in YPAD and 10-fold serial dilutions were spotted on YPAD media at 23, 30, 34 and 37°C to test for viability. (**B**) Quantitative measurement of single-stranded DNA (QAOS) in the indicated (arrows) subtelomeric regions of wild-type, *stn1-Nt*, *stn1-63* and *cdc13-1* strains (YAP46, YAP42, YAP73 and DLY1108, respectively). Samples were collected at the permissive temperature (24°C; $t=0$) and after 180 min of growth at the non-permissive temperature (37°C; $t=180$).

A

B

C

EST2
stn1-Nt

est2D670A
stn1-Nt

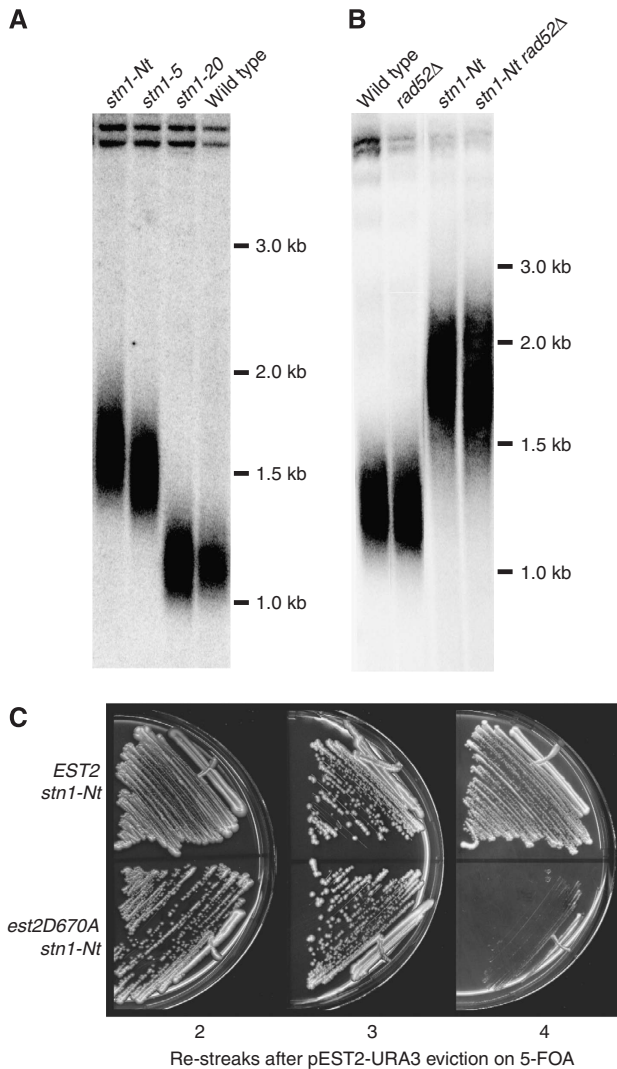2   3   4

Re-streaks after pEST2-URA3 eviction on 5-FOA

Figure 3 Analysis of telomere length in different *stn1* mutant strains. (**A**) Southern blot of *Xho*I-digested genomic DNA hybridized with a Y′ probe. The *stn1-Nt*, *stn1-5* and *stn1-20* strains (YAP73, YAP70 and YAP71, respectively) were streaked five times prior to DNA extraction. (**B**) Telomere elongation in the presence of the *stn1-Nt* allele is recombination independent (*rad52Δ* = YAP162; *stn1-Nt rad52Δ* = YAP172) and (**C**) telomerase dependent (*stn1-Nt est2D670A* =YAP115).

telomeric chromatin. We thus used a ChIP assay to measure binding of a 13 × Myc epitope-tagged version of Stn1 (Stn1–Myc) as a function of cell cycle progression. In addition, we performed these experiments in a pair of strains that differ in TG tract length at a single marked telomere (Bianchi and Shore, 2007a, b), thus allowing us to test the idea that binding of the negative regulator Stn1 might be increased at longer TG tracts, where telomerase action is disfavoured (Teixeira *et al*, 2004). In one strain, Cre–LoxP recombination leads to a shortened TG tract at an engineered Chr. V-R telomere (Figure 4B), whereas in a control strain the recombination event leaves the same Chr. V-R telomere tract unchanged (Figure 4A). In both strains, Stn1–Myc binding at a different telomere (Chr. VI-R) served as an internal control. Cre-mediated recombination was induced by the addition of galactose to cell cultures that were immediately afterwards arrested in the G1 phase of the cell cycle by the addition of

mating pheromone (α factor). Following release of cells into a synchronous cell cycle by removal of α factor, protein binding (Stn1–Myc) was measured by ChIP at 20-min intervals.

This experiment demonstrated that Stn1 associates specifically with both telomeres examined, with a clear peak of binding occurring at 100 min following α factor release (Figure 4C), which under these conditions corresponds to late S phase (data not shown). Similar binding profiles have been observed by us and others for epitope-tagged versions of Cdc13, Est1 and Est2 (Taggart *et al*, 2002; Schramke *et al*, 2004; Takata *et al*, 2005; Bianchi and Shore, 2007a, b). Significantly, we observed no temporal or quantitative difference between Stn1 association at the shortened Chr. V-R telomere compared with either the internal Chr. VI-R telomere or the unshortened V-R telomere in the control strain. A similar result is observed for Cdc13, whereas both Est1 and Est2 binding is markedly increased (∼2-fold) at a shortened telomere (Bianchi and Shore, 2007b).

Stn1 C terminus influences the efficiency of telomerase action at an elongating telomere

To analyse further the dynamics of telomerase-dependent TG addition in the *stn1-Nt* mutant, we turned to a *de novo* telomere formation assay (Figure 5A). We and others have shown previously that the presence of an 80-bp telomeric repeat sequence adjacent to a DNA double-strand break (DSB; generated by induced expression of the HO endonuclease) is sufficient to provoke rapid and efficient telomerase-dependent TG-repeat addition at the break (Diede and Gottschling, 1999, 2001; Bianchi *et al*, 2004). This telomere-'healing' reaction depends on both Cdc13 and Est1, and in particular on an interaction between these two proteins characterized genetically by Lundblad and colleagues (Evans and Lundblad, 1999; Pennock *et al*, 2001) and Bianchi *et al* (2004). To avoid complications that might arise from variable expression of plasmid-borne copies of mutant *stn1-Nt*, we have performed these experiments in strains where the *stn1-Nt* mutant allele is integrated into the chromosome (indicated as *stn1-Nt-i*). It should be noted that in these experiments both the wild-type and the truncated *STN1* gene are expressed from the strong *ADH1* promoter.

We thus decided to compare, in wild-type and *stn1-Nt-i* strains, the efficiency and extent of telomere elongation, as well as the binding of Cdc13 and Est1 proteins to the forming telomere. As shown in Figure 5B, the wild type and mutant display remarkably different behaviour in this telomere-'healing' assay. Thus, the smear of lower mobility species (elongating ends) appears earlier in the mutant, where the overall rate of end elongation is clearly higher. Furthermore, the band resulting from the HO cut ('C' in Figure 5B) clearly disappears at a higher rate in the mutant cells (∼2-fold higher in several independent experiments, data not shown), consistent with an increase in the frequency of telomerase action. An alternative explanation of this increased rate would be that the TG ends in the *stn1-Nt-i* mutant cells are more prone to degradation, perhaps through 5′ end resection. To test this idea, we first quantified the efficiency of stable telomere formation in mutant and wild-type cells, using a simple biological assay (measurement of Ade + , Lys− colonies by replica plating). By this measure, telomere formation in the mutant cells is 80% that of wild type (data not shown). We next used a ChIP assay to measure
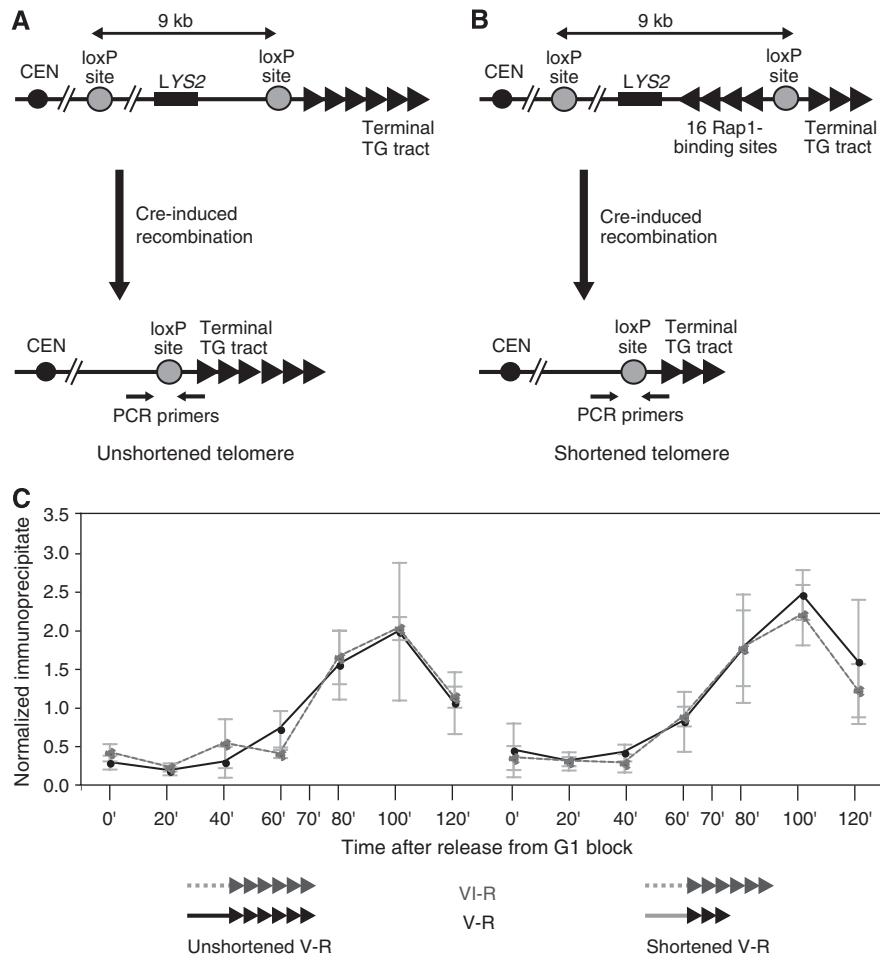
Figure 4 A system for the generation of a single shortened telomere on Chr. V-R to measure the association of Myc-tagged Stn1. (**A**, **B**) Cre-induced recombination of the terminal part of Chr. V-R generates an unshortened telomere (A) or a shortened telomere (B) depending on the absence or presence of 16 Rap1-binding sites immediately internal to the distal LoxP site. (**C**) Quantification by chromatin immunoprecipitation (ChIP) of the association of Stn1–Myc to the unshortened (left) and shortened (right) Chr. V-R telomere (continuous line), normalized to the signal obtained at the reference Chr. VI-R telomere (dashed line) in each strain. Data shown are the average of three independent experiments.

RPA binding at the break site, which serves as a highly sensitive measure of ss DNA production following HO cutting (Hirano and Sugimoto, 2007). This assay revealed a slight ($\sim$2-fold) increase in the mutant relative to the wild type at the TG-80 end of the break, though to levels still 10-fold below that of an end not containing TG repeats (Supplementary Figure S5). Taken together, these data suggest that the TG-flanked HO cut site in the *stn1-Nt-i* mutant cells may be more permissive to both exonucleolytic attack and telomerase action, though the precise rate of either is at present difficult to determine.

To address the mechanism by which the Stn1 C terminus negatively regulates telomere elongation, we used the ChIP assay to quantify the level of both Cdc13 and Est1 binding at the forming telomere (Bianchi *et al*, 2004; Negrini *et al*, 2007). As shown in Figure 5C and D, binding of both Cdc13 and Est1 at the elongating TG-80 end was approximately two-fold higher in an *stn1-Nt-i* strain than in a wild-type strain. This increase in Cdc13 and Est1 binding might result from the action of telomerase, which generates additional TG-repeat sequence, and thus more potential binding sites for Cdc13 (and, indirectly, Est1). We therefore conducted similar ChIP experiments in strains expressing a catalytically inactive

version of Est2 (est2D670A), where TG-repeat addition at the break does not occur (Supplementary Figure S6). However, we found that Cdc13 binding remained high in the *stn1-Nt-i* strain, relative to the *STN1* wild-type control (as did that of Est1), even in the absence of telomerase action (Supplementary Figure S3), suggesting that it might be largely due to the apparent increase in ss DNA at the break site in these cells.

Continuous telomere elongation in stn1-Nt cells
Mutations in other negative regulators of telomere elongation, such as Rif proteins, result in a new steady-state telomere length (Hardy *et al*, 1992; Wotton and Shore, 1997). This new equilibrium is reached relatively rapidly (probably <25 generations) due to active telomerase-dependent elongation (Diede and Gottschling, 1999; Levy and Blackburn, 2004). Surprisingly, we found instead that TG repeat addition an *stn1-Nt* strain continues for at least $\sim$340 generations (17 re-streaks, $\sim$20 generation per streak), thus resulting in telomeres with TG tracts that measured $\sim$4 kb in length (Figure 6A). Remarkably, the average rate of this continuous elongation was about 130 bp per re-streak and appeared to be largely independent of TG tract length. Thus,
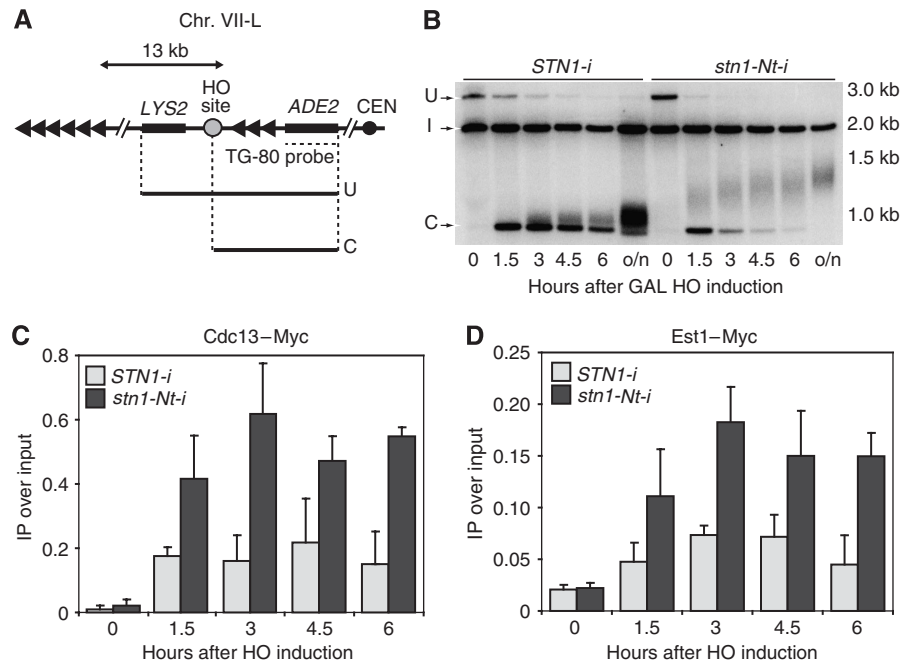
Figure 5 Increased efficiency of telomerase elongation at an HO-induced DNA double-strand break (DSB) in strains carrying a chromosomal *stn1-Nt* allele. (**A**) Schematic representation of the modified subtelomeric region of Chr. VII-L. (**B**) Southern blot monitoring the healing at Chr. VII-L after DSB induction 'I' indicates a band from the *NMD5* locus that serves as an internal loading standard, 'U' denotes the uncut fragment hybridizing to an *ADE2* probe and 'C' indicates the band resulting from 'U' after HO cleavage. Telomere formation at the DSB was detectable as a smeared band just above the 'cut' (C) fragment (see Materials and methods for details). (**C, D**) Analysis by ChIP of the binding of Cdc13–Myc (*STN1-i* = YAP210; *stn1-Nt-i* = YAP211) and Est1–Myc (*STN1-i* = YAP214, *stn1-Nt-i* = YAP215) following induction of a DSB.

even at the later generations, the elongation rate is maintained at the same average rate as in the earlier ones (~7 bp per cell division). In addition, we note that late generation cells (200 and beyond) display apparent amplification of the short Y' element as well as an increase in a sub-population of shorter telomeres that may be the result of telomere rapid deletion (Bhattacharyya and Lustig, 2006).

Our two-hybrid analysis indicates that the C- and N-terminal truncations of Stn1 can independently interact with Ten1 and Cdc13–Pol12, respectively (Figure 1). This observation led us to investigate whether the apparently unlimited telomere elongation caused by the absence of Stn1 C terminus could be reversed by the co-expression of the C-terminal portion of Stn1. To test this hypothesis, a multicopy plasmid carrying the Stn1 C-terminal domain driven by a *GAL1* promoter was constructed. The galactose-induced overexpression of this portion of Stn1 is lethal in the *stn1-Nt* mutant strain (as in an *STN1* wild-type strain). However, under repressing condition (growth on glucose-containing medium), where the Stn1 C-terminal fragment is weakly expressed (data not shown), cell viability was unaffected and, as shown in Supplementary Figure S4, the telomere elongation defect conferred by the *stn1-Nt* mutation was significantly reduced. This result indicates that the Stn1 C terminus, which can bind to Cdc13 but not to Ten1, is alone able to negatively regulate telomere elongation.

MRX and Tel1 are required for continuous telomere elongation in stn1-Nt cells

To understand better the role of the Stn1 C-terminal domain in telomerase regulation, we generated strains carrying the *stn1-Nt* allele in combination with mutations in other factors

involved in telomere length regulation. Several studies have shown that telomere length equilibrium results from a balance of positive and negative regulatory networks. Mutations in *RIF1* and *RIF2*, part of the so-called 'protein-counting' mechanism (Marcand *et al*, 1997, 1999), lead to telomere over-elongation, whereas mutations in components of the MRX complex (Mre11, Rad50 or Xrs2) or Tel1 (the yeast ATM homologue) have been shown to result in short, but stable telomeres (reviewed in McEachern *et al*, 2000). Genetic epistasis analysis suggests that MRX and Tel1 act in a common pathway downstream from both Rif1 and Rif2 (Ritchie and Petes, 2000; Chan *et al*, 2001; Grossi *et al*, 2004). As shown Figure 6C and D, gene disruption of either *RAD50* or *TEL1* caused telomere shortening, whereas disruption of *RIF2* induced telomere lengthening, as previously reported. Notably, however, both *stn1-Nt tel1-Δ* and *stn1ΔNt rad50-Δ* double mutants, and an *stn1-Nt tel1-Δ rif2-Δ* triple mutant, had telomeres that were longer than the wild-type strain, indicating that the *stn1-Nt* can completely reverse the telomere shortening effect of both *tel1-Δ* and *rad50-Δ*. Nevertheless, both *tel1-Δ* and *rad50-Δ* abolish the continuous elongation phenotype conferred by *stn1-Nt*. By contrast, *stn1-Nt sml1-Δ mec1-Δ* cells (lacking the yeast ATR homologue Mec1) do display telomere over-elongation (Figure 6B). Finally, the double mutant *stn1-Nt rif2-Δ* shows very long and heterogeneous telomeres.

Discussion

The results described here demonstrate that the telomere capping and telomere length control functions of Stn1 are to a large extent executed by two independent domains that can function
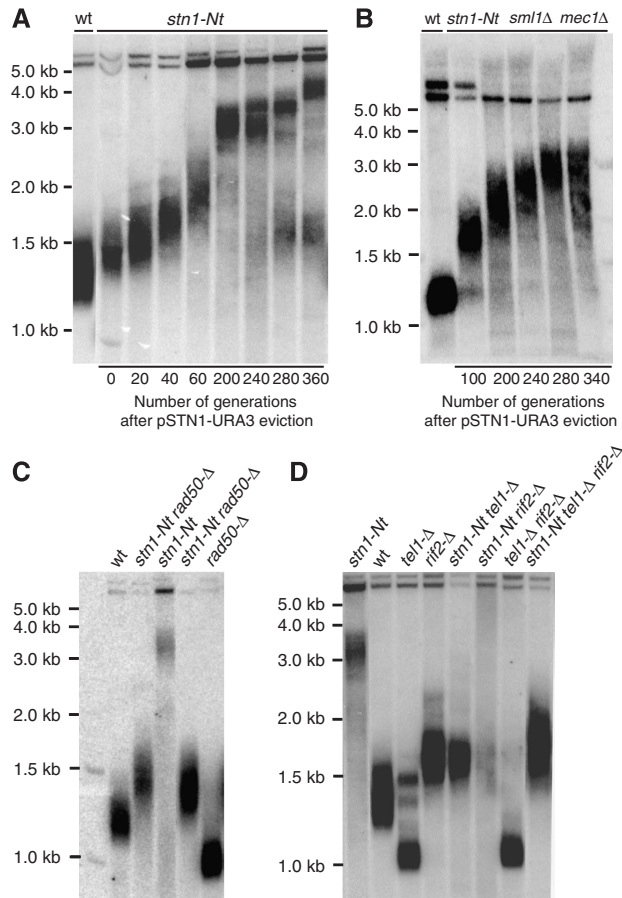
Figure 6 Telomere length and genetic interaction analysis of *stn1-Nt*. (**A**, **B**) Southern blot of *Xho*I-digested genomic DNA hybridized with a Y′ probe. Following YCpLac33-STN1 eviction, cells were grown on YPAD medium at 30°C for the indicated number of generations before preparation of genomic DNA. (**C**, **D**) Southern blot analysis, using a Y′ probe, of *Xho*I-digested genomic DNA from the indicated single-, double- and triple-mutant strains. After tetrad dissection of a heterozygous diploid strain, the indicated mutant segregants were re-streaked at least 15 times on YPD medium at 30°C prior to DNA preparation.

separately from each other. These functional domains are associated with discrete protein–protein interactions: the Stn1 N terminus interacts with Ten1 to cap the telomere, whereas the C terminus of Stn1 interacts with both Cdc13 and Pol12, with the Cdc13 interaction having a critical function in the regulation of telomerase action. One possible caveat to these conclusions is that the *stn1-Nt* mutant allele must be expressed from a strong promoter (*ADH1*) for the cells to be viable, as has also been observed by Petreaca *et al* (2007). Although it is difficult to rule out the possibility that this high level of expression might in and of itself influence the *stn1-Nt* phenotype, we note that *de novo* telomere formation and equilibrium telomere length are similar or identical when comparing wild-type *STN1* expressed from either its native promoter or the *ADH1* promoter (Figure 5 and data not shown).

Capping function of the Stn1 N terminus

Our data show that an N-terminal domain (amino acids 1–281) of Stn1 fulfills its essential function of telomere end protection, consistent with recent reports on both the *S. cerevisiae* protein (Gao *et al*, 2007; Petreaca *et al*, 2007) and its homologue in the related yeast *K. lactis* (Iyer *et al*, 2005).

This capping function of Stn1 may derive solely from its ability to interact with Ten1, as the capping defect in *stn1-154*, which we show is due to an Asp to Glu substitution at amino acid 99, is associated with an abolished Ten1 interaction and is suppressed by fusion of the mutant Stn1 protein with Ten1 (Grandin *et al*, 2001). Interestingly, the failure of stn1-Nt to interact with Cdc13 in a two-hybrid assay (Figure 1) suggests that telomere capping may not require a direct interaction between Stn1 and Cdc13, as proposed previously (Pennock *et al*, 2001). Delivery of stn1-Nt to telomeres may also be promoted by Ten1, which itself interacts with Cdc13 (Grandin *et al*, 2001; Gao *et al*, 2007), and with the telomeric TG-rich single-strand overhang, through its own OB fold (Gao *et al*, 2007). An alternative interpretation of our data is that stn1-Nt and Ten1 are able to bind the telomere tip independently of Cdc13, when overexpressed, thus bypassing the normal Cdc13 requirement for telomere capping (Petreaca *et al*, 2006). Consistent with this possibility, an OB fold-containing region of stn1-Nt (amino acids 69–199) and full-length Ten1 (which also contains an OB fold) have recently been shown to bind specifically *in vitro* to TG-containing ss DNA (Gao *et al*, 2007).

Our finding that Pol12 fails to interact with the Stn1 N terminus in two-hybrid assays is in contradiction to *in vitro* studies reported previously (Petreaca *et al*, 2006). We do not know the reason for this discrepancy, but would point out that it is unlikely to be due to a trivial artefact, because the GAD–stn1-Nt hybrid used in our experiments is functional: it complements an *STN1* deletion for growth and interacts well with GBD-Ten1. In any event, we have confirmed by a GST–Pol12 pull-down assay that both the Stn1 N and C termini, expressed independently in yeast, can bind to Pol12 (Supplementary Figure S7). We would therefore suggest that both halves of Stn1 contribute *in vivo* to Pol12 binding. As our conclusion that the Stn1–Cdc13 interaction (and not the Stn1–Pol12 interaction) has a predominant function in telomere length regulation is based on a defect in stn1-20 binding to Pol12, we have tested this *in vitro*. We observe a reduction of at least 25% in binding of the mutant compared with wild type (Supplementary Figure S7). This result is consistent with the two-hybrid findings, though weaker in magnitude, perhaps because the *in vitro* studies were performed with full-length Stn1 protein. Taken together, the data support our conclusion that the Stn1–Cdc13 interaction has a predominant function in telomere length regulation, and highlight the need for further studies of the Pol12 interaction with Stn1, and its functional consequences, particularly in telomere capping (Grossi *et al*, 2004; Petreaca *et al*, 2006).

Finally, it is worth noting that Iyer *et al* (2005) have described a different point mutation in the conserved N terminal region of the *K. lactis* Stn1 protein that leads to high levels of recombinogenic telomere elongation, reminiscent of that seen in human tumour cells with an active alternative lengthening of telomeres pathway. It will be interesting to see whether this or other mutations in the *S. cerevisiae* Stn1 protein will have similar effects.

Telomerase regulation and coordination of C-strand synthesis by Stn1

Telomere length homoeostasis is achieved through a balance between telomere elongation and shortening. Recent studies by Lingner and colleagues (Teixeira *et al*, 2004) show that

telomeres in budding yeast are not elongated by telomerase at every cell cycle, and that the probability of elongation is inversely related to TG tract length. These and other data indicate that a mechanism(s) operates in *cis* that can sense TG tract length and use this information to control telomerase access and/or activity. Rap1 and its interacting Rif factors appear to be sensors of telomere length and negative regulators of telomerase action, whereas Cdc13, which is required to recruit telomerase to chromosome ends through an interaction with Est1, would appear to be an important regulatory target. Indeed, several recent studies indicate that the recruitment of the telomerase holoenzyme (including Est1) is a key step regulated by telomere tract length (Bianchi and Shore, 2007b; Hector *et al*, 2007; Sabourin *et al*, 2007).

Data presented here suggest that the Stn1 C terminus, which interacts with Cdc13 in two-hybrid assays, has a key and perhaps unique function as a negative regulator of telomerase action. Thus, cells lacking the Stn1 C terminus display an unusual telomere elongation phenotype, where elongation occurs at a constant rate (essentially independent of TG tract length) for at least 350 generations, yielding extremely elongated telomeres that are unusually homogenous in length. This phenotype is quite unlike that of cells lacking both Rif1 and Rif2 or the Rap1 C terminus, where elongation is more rapid and telomere length in a population of cells is extremely heterogeneous. Furthermore, in cells deleted for either *RIF1* or *RIF2*, telomeres reach a new average length, unlike what is observed in *stn1-Nt* mutants. Significantly, the effect of *stn1-Nt* on telomere elongation is additive with that of *rif2-Δ*, which has been shown to increase the probability of telomerase action but not its processivity (Teixeira *et al*, 2004), arguing either that *stn1-Nt* affects telomerase accessibility through a different pathway than Rif2, or that it effects the activity (processivity) of telomerase already bound at the chromosome end. We propose that the more rapid elongation that occurs following HO cutting in the *stn1-Nt* strain reflects repeated rounds of telomerase binding and synthesis as these cells remain blocked in G2/M during the course of the experiment (Negrini *et al*, 2007), suggesting that the nascent telomere is either more accessible to telomerase, or in a state that more efficiently promotes telomerase synthesis once bound (or both). Although we cannot rule out an increase in telomerase processivity brought about by the deletion of the Stn1 C terminus, we note that telomeres in normally cycling *stn1-Nt* cells elongate at a modest pace (approximately 7 bp per cell division) and maintain a remarkably tight length distribution in the population, arguing against the idea that telomerase addition becomes unregulated under these conditions.

Our two-hybrid and ChIP data, together with the earlier findings of Chandra *et al* (2001), are consistent with a model in which the Stn1 C terminus and Est1 compete directly for Cdc13 binding. One caveat to this conclusion, though, is our finding that Cdc13 association increases concomitantly with that of Est1 in *stn1-Nt* strains. We are thus unable to determine whether increased Est1 binding is simply a consequence of increased Cdc13 telomere association or instead due to increased accessibility of telomere-bound Cdc13 to Est1 (or both). Why does Cdc13 telomere association appear to increase as a consequence of deletion of the Stn1 C terminus? Our data suggest that this might be due to the slight increase in ss TG-rich DNA detected in the *stn1-Nt* strain, which would increase the number of potential Cdc13-binding sites there.

This increased single-strandedness might stem from weakened (or altered) recruitment of the polymerase α–primase complex to telomere ends, which is also promoted by the Stn1 C terminus, in this case through its interaction with Pol12 (Figure 1A; Grossi *et al*, 2004), leading to defective C-strand synthesis at or near the telomere terminus. Alternatively, the absence of the Stn1 C terminus in the CST complex may expose an additional region of Cdc13 to the ss overhang, increasing our ability to crosslink the protein to telomeric sequences. These two explanations are not mutually exclusive and additional work will be required to resolve this issue.

Stn1 and the regulatory system that monitors TG tract length

As pointed out above, several recent studies have demonstrated increased association of both telomerase holoenzyme (Est1 and Est2 subunits) and Tel1 kinase, but not Cdc13, at short telomeres (Bianchi and Shore, 2007b; Hector *et al*, 2007; Sabourin *et al*, 2007). One explanation for these observations is that the telomere length signal (still poorly defined, but presumably generated by the Rap1–Rif complex) modulates Tel1 binding, which in turn affects the phosphorylation state of the CST complex and, consequently, its ability to recruit telomerase. Consistent with this scenario, Tel1 kinase can phosphorylate Cdc13 *in vitro* at residues within the Cdc13 telomerase recruitment domain that are required for telomere length maintenance *in vivo* (Tseng *et al*, 2006).

We show here that Stn1 telomere association, similar to that of Cdc13, is quantitatively indistinguishable at short versus long telomeres. This finding suggests that the negative effect of the Stn1 C terminus on telomerase action is either constant (i.e. independent of telomere length) or modulated by telomere length through a modification of Stn1 at the telomere, rather than a change in the amount of protein associated there. Our finding that *stn1-Nt* strains display considerable telomere elongation even in the absence of Tel1 function (or that of the MRX complex; Figure 6), suggests that the Stn1 C terminus exerts an effect in parallel with Tel1 to control telomere length, probably by directly modulating telomerase association. The data also raise the possibility that the Stn1 C terminus is itself one of several downstream targets of the Tel1 kinase. In this scenario, phosphorylation of Stn1 would be promoted at short telomeres and would weaken its interaction with Cdc13, thus promoting Est1 (and telomerase enzyme) association. However, we found that mutation of three potential Tel1 target sites in Stn1 has no detectable effect on telomere length (unpublished data), arguing against this model. We thus favour a model in which Stn1 provides a constitutive inhibitory signal, independent of telomere tract length, whose effectiveness is modulated by changes in its partner protein Cdc13, and possibly other factors.

In summary, the data presented here suggest that the Stn1 C terminus, through an interaction with Cdc13, has a direct function in inhibiting telomerase association with the chromosome end, a process that recent work suggests is highly dynamic (Chang *et al*, 2007). Our analysis suggests that this 'brake' on telomere elongation exerted by the Stn1 C terminus is counteracted by the positive arm of a 'protein-counting' telomere length-regulatory mechanism involving the Tel1 kinase. Although the latter mechanism is modulated by

telomere length (Bianchi and Shore, 2007b; Sabourin *et al*, 2007), we show that the Stn1 inhibition is likely not to be. We note that recent work in mammalian cells indicates that increasing telomerase levels also promotes a constant, tract length-independent telomere elongation phenotype (Cristofari and Lingner, 2006). The proposed similarity of the CST complex to the well-studied RPA complex (Gao *et al*, 2007) raises the interesting possibility that structural changes in CST, analogous to those proposed to occur in RPA (Fanning *et al*, 2006), might have an important function in regulating its interactions with both telomerase and the polymerase α–primase complex. A challenge for future studies will be to design experiments that allow one to characterize directly CST, telomerase holoenzyme and polymerase α–primase interactions *in vitro*, and, ultimately, during the dynamic

process of telomere replication in living cells. The recent discovery of Stn1-like proteins in many organisms (Gao *et al*, 2007; Martin *et al*, 2007), including mammals, suggests that the findings reported here may have relevance beyond the budding yeasts.

Materials and methods

Yeast strains and plasmids
The yeast strains used in this study are listed in Table I, and are isogenic to W303 (Thomas and Rothstein, 1989), apart from specific point mutations or gene deletion/disruptions, or where otherwise indicated. Double-mutant strains were obtained by crossing of single-mutant strains followed by sporulation and tetrad dissection, unless indicated otherwise. Growth and manipulation of yeast were carried out according to standard procedures (Adams *et al*, 1997).

Table I Yeast strains used in this study

Name	Relevant genotype	Reference
W303-1A	*MAT* **a** *ade2-1 trp1-1 can1-100 leu2-3, 112his3-11, 15 ura3-1rad5-535*	Thomas and Rothstein (1989)
W303-1B	*MAT* **a** *ade2-1 trp1-1 can1-100 leu2-3, 112 his3-11, 15 ura3-1rad5-535*	Thomas and Rothstein (1989)
PJ69-4A	*MAT* **a** *trp1-901 leu2-3,112 ura3-52 his3-200 gal4Dgal80D LYS''*::*GAL1-HIS3 GAL2-ADE2 met2*::*GAL7-lacZ*	James *et al* (1996)
CTY10-5D	*MAT* **a** *ade2 trp1-901 leu2-3, 112 his3-200 gal4 gal80 URA3*::*lexA op-lacZ*	Bartel and Fields (1995)
YAB53	W303 (*RAD5, lys2*), *mata*::*loxP, leu2*::*pGALHO, mnt2*::*LYS2*	Bianchi *et al* (2004)
DLY1108	*MAT* **a** *cdc13-1*	Booth *et al* (2001)
YAP34	YAB53, *CDC13*-Myc13::*HIS3*, VII-L::*ADE2-Dbp(amp7)*-TG-80-HO site	This study
YAP41	*MAT* **a** *stn1*_LoxP + *pGADstn1-154-LEU2*	This study
YAP42	*MAT* **a** *stn1*_LoxP + *pGADstn1-63-LEU2*	This study
YAP46	*MAT* **a** *stn1*_LoxP + YCp-*STN1-URA3*	Grossi *et al* (2004)
YAP70	*MAT* **a** *stn1*_LoxP + *pGADstn1-5-LEU2*	Grossi *et al* (2004)
YAP71	*MAT* **a** *stn1*_LoxP + *pGADstn1-20-LEU2*	Grossi *et al* (2004)
YAP73	*MAT* **a** *stn1*_LoxP + *pGADstn1-Nt-LEU2*	Grossi *et al* (2004)
YAP100	YAB53, *EST1*-Myc13::*HIS3*, VII-L::*ADE2-Dbp(amp7)*-TG-80-HO site	This study
YAP115	YAB53, *est2D670A, CDC13*-Myc13::*HIS3*, VII-L::*ADE2-Dbp(amp7)*-TG-80-HO site	This study
YAP117	YAB53, *est2D670A, EST1*-Myc13::*HIS3*, VII-L::*ADE2-Dbp(amp7)*-TG-80-HO site	This study
YAP130	YAB53, *CDC13*-Myc13::*HIS3*, stn1::*KanR*, pGAD*stn1-Nt-TRP1*,VII-L::*ADE2-Dbp(amp7)*-TG-80-HO site	This study
YAP131	YAB53, *EST1*-Myc13::*HIS3*, stn1::*KanR*, pGAD*stn1-Nt-TRP1*,VII-L::*ADE2-Dbp(amp7)*-TG-80-HO site	This study
YAP150	*MAT* **a** *stn1*_LoxP + *pGADstn1-Nt-LEU2*, adh4::*URA3*	This study
YAP151	*MAT* **a** *stn1*_LoxP + *pGADstn1-63-LEU2*, adh4::*URA3*	This study
YAP152	*MAT* **a** *sir2*::*HIS3, adh4*::*URA3*	This study
YAP153	*MAT* **a** *adh4*::*URA3*	This study
YAP162	*MAT* **a** *rad52*::*KanR*	This study
YAP170	*MAT* **a** *stn1*_LoxP + *pGADstn1-Nt-LEU2*	This study
YAP171	*MAT* **a** *stn1*_LoxP + *pGADstn1-Nt-LEU2*, rad50::*KanR*	This study
YAP172	*MAT* **a** *stn1*_LoxP + *pGADstn1-Nt-LEU2*, rad52::*KanR*	This study
YAP175	*MAT* **a** *tel1*::*TRP1*	This study
YAP176	*MAT* **a** *stn1*_LoxP + *pGADstn1-Nt-LEU2*, tel1::*TRP1*	This study
YAP177	*MAT* **a** *stn1*_LoxP + *pGADstn1-Nt-LEU2 rif2*::*HIS3*	This study
YAP178	*MAT* α *te1*::*TRP1, rif2*::*HIS3*	This study
YAP179	*MAT* **a** *stn1*_LoxP + *pGADstn1-Nt-LEU2, te1*::*TRP1, rif2*::*HIS3*	This study
YAP180	*MAT* α, *rif2*::*HIS3*	This study
YAP189	YAB53, *est2D670A, CDC13*-Myc13::*HIS3*, stn1::*KanR*, pGAD*stn1-Nt-TRP1*, VII-L::*ADE2-Dbp(amp7)*-TG-80-HO site	This study
YAP190	YAB53, *est2D670A, EST1*-Myc13::*HIS3*, stn1::*KanR*, pGAD*stn1-Nt-TRP1*, VII-L::*ADE2-Dbp(amp7)*-TG-80-HO site	This study
YAP204	YAB53, *CDC13*-Myc13::*HIS3*, stn1::*KanR*, YCpLac33-*STN1-URA*,VII-L::*ADE2-Dbp(amp7)*-TG-80-HO site	This study
YAP205	YAB53, *EST1*-Myc13::*HIS3*, stn1::*KanR*, YCpLac33-*STN1-URA*,VII-L::*ADE2-Dbp(amp7)*-TG-80-HO site	This study
YAP210	YAB53, *CDC13*-Myc13::*HIS3*, stn1::*KanR*, GAD-*STN1-HA-i*::*TRP1*,VII-L::*ADE2-Dbp(amp7)*-TG-80-HO site	This study
YAP211	YAB53, *CDC13*-Myc13::*HIS3*, stn1::*KanR*, GAD-*stn1-Nt-HA-i*::*TRP1*,VII-L::*ADE2-Dbp(amp7)*-TG-80-HO site	This study
YAP213	YAB53, *EST1*-Myc13::*HIS3*, stn1::*KanR*, GAD-*STN1-HA-i*::*TRP1*,VII-L::*ADE2-Dbp(amp7)*-TG-80-HO site	This study
YAP214	YAB53, *EST1*-Myc13::*HIS3*, stn1::*KanR*, GAD-*stn1-Nt-HA-i*::TRP1,VII-L::*ADE2-Dbp(amp7)*-TG-80-HO site	This study
YG145	W303 *MAT* α, *yku70*::*LEU2*	This study
YSG376	YAB53, VII-L::*ADE2-Dbp(amp7)* λ *DNA-HO* site	This study
YAP220	W303 *MAT* α, *GAD-STN1-HA-i*	This study
YAP221	W303 *MAT* α, *GAD-stn1-20-HA-i*	This study
YAP222	W303 *MAT* α, *GAD-stn1-Nt-HA-i*	This study
YAP223	W303 *MAT* α, *GAD-stn1-Ct-HA-i*	This study

Table II Plasmids used in this study

Name	Description	Reference
YCpLac33-STN1	*CEN, URA3, STN1*, native promoter	Grandin *et al* (1997)
pACT2-STN1-HA	2 μm, *LEU2, GAD-STN1, ADH1* promoter	Grandin *et al* (1997)
YCp1ll-stn1-154	*CEN, LEU2, stn1-154*, native promoter	Grandin *et al* (2001)
pGAD-stn1-5-HA	2 μm, *LEU2, GAD-stn1-5, ADH1* promoter	This study
pGAD-stn1-20-HA	2 μm, *LEU2, GAD-stn1-20, ADH1* promoter	This study
pstn1-63	*CEN, LEU2, stn1-63*, native promoter	This study
pGAD-stn1-63-HA	2 μm, *LEU2, GAD-stn1-63, ADH1* promoter	This study
pGAD-stn1-Nt-HA	2 μm, *LEU2, GAD-stn1-Nt, ADH1* promoter	This study
pGAD-stn1-Ct-HA	2 μm, *LEU2, GAD-stn1-Ct, ADH1* promoter	This study
pGAL-stn1-Ct	2 μm, *LEU2, GAL prom-stn1-Ct*	This study
pAS2-TEN1	2 μm, *TRP1, GBD-TEN1, ADH1* promoter	Grandin *et al* (2001)
pLexA-pol12-Nt	2 μm, *TRP1, pLexA-pol12-Nt, ADH1* promoter	Grossi *et al* (2004)
GBD-CDC13	2 μm, *TRP1, pGBD-CDC13, ADH1* promoter	Bianchi *et al* (2004)
pGBD	2 μm, *TRP1, pGBD, ADH1* promoter	James *et al* (1996)
pGAD	2 μm, *LEU2, GAD, ADH1* promoter	Grandin *et al* (1997)
PLexA	2 μm, *TRP1, LexA, ADH1* promoter	Bartel and Fields (1995)
pGAD-STN1-HA-i	Integrative, *TRP1, ADH1* promoter, *GAD-STN1*	This study
pGAD-stn1-Nt-HA-i	Integrative, *TRP1, ADH1* promoter, *GAD-Stn1-Nt*	This study
pGAD-stn1-Ct-HA-i	Integrative, *TRP1, ADH1* promoter, *GAD-Stn1-Ct*	This study
pGAD-stn1-20-HA-i	Integrative, *TRP1, ADH1* promoter, *GAD-Stn1-20*	This study

CDC13, *EST1* and *STN1* were tagged using PCR products generated from pFA6a-13Myc-His3MX6 (Longtine *et al*, 1998). All strains used for the telomere healing assay are derived from W303-based YAB53 (Bianchi *et al*, 2004), and carry a deletion of the HO site at the *MAT* locus; a galactose-inducible copy of the HO endonuclease gene at the *LEU2* locus; a cassette at the subtelomeric region of Chr. VII-L, between the *ADH4* and *MNT2* genes, containing the *ADE2* gene, a sequence from the mouse *Dbp* gene (amplicon 7), a 80-bp TG-repeat sequence from a native telomere and the HO cut site (Figure 5A). The *STN1* gene was deleted in a diploid YAB53 strain using a PCR-generated *KanMX* (G418 resistance) cassette (Longtine *et al*, 1998).

The plasmids used in this study are listed in Table II. pGAD-stn1-Nt was generated by subcloning an *Nco*I–*Eco*RI fragment from pACT2-STN1 (Grandin *et al*, 1997) in *Nco*I–*Eco*RI-digested pGAD (Moretti *et al*, 1994). pGAD-stn1-Ct was generated by subcloning the *Eco*RI fragment from pACT2-STN1 in *Eco*RI-cleaved pGAD. The pstn1-63 plasmid was generated by subcloning the *Nco*I–*Bsm*I fragment from YCp1ll-stn1-154 (Grandin *et al*, 2001) in *Nco*I–*Bsm*I-digested YCpLac33-STN1 (Grandin *et al*, 1997). Integrative plasmids (*GAD-stn1-HA-i*) carrying the different *GAD-stn1-HA* alleles were generated by cloning a *Sap*I–*Xho*I fragment from the pGAD-stn1-HA plasmids into *Sap*I–*Xho*I digested pRS304. These constructs were digested with *Eco*RV and integrated in the *TRP1* locus of yeast strains deleted for endogenous copy of *STN1* and kept alive with YCpLac33-STN1-URA plasmid. The cells were then streaked on 5-FOA to evict the YCpLac33-STN1-URA plasmid.

PCR mutagenesis (Cadwell and Joyce, 1994) was used to generate *STN1* C terminus mutations, and gap repair was used to generate pACT2-STN1 mutant plasmids. *Eco*RI-linearized pACT2-STN1 was co-transformed in the CTY10-5D yeast strain (already containing pLexA-Pol12-Nt) together with the mutagenized *STN1* C-terminal PCR product. After transformation, cells were grown in liquid YAPD for 4 h and then plated on SC-Trp-Leu solid media containing X-gal.

Two-hybrid assays

The yeast two-hybrid assays were performed using CTY10-5D (Bartel and Fields, 1995) or PJ69-4A (James *et al*, 1996) strains harbouring pLexA/pGAD or pGBD/pGAD fusion plasmids, respectively. Strains containing the test plasmids were grown for 24 h in SC-Trp-Leu liquid medium, and β-galactosidase was measured by liquid assay (Moretti *et al*, 1994).

Telomere Southern blots

For Southern blot analysis of telomere length, yeast genomic DNA was isolated from overnight cultures and 2 μg was digested with *Xho*I. DNA fragments were separated by electrophoresis in 1.0% agarose gels (TBE 0.5 ×), transferred to HyBond N + membranes, and hybridized with a random-primed, radiolabelled Y′ probe (Craven and Petes, 1999) by standard procedures. The membranes

were then autoradiographed with a PhosphorImager (Bio-Rad Molecular Imager FX).

Southern analysis of DSB induction and telomere healing assay

For galactose induction of a DSB, overnight saturated cultures were diluted in YPE lactate to a final concentration of 5×10^6 cells/ml and grown for additional 3 h at 30°C. The HO endonuclease was then induced by the addition of galactose to a final concentration of 2%. DNA was isolated from 40 ml of cell culture and digested with *Eco*RV. DNA fragments were separated by electrophoresis in 0.8% agarose gels, transferred to HyBond N + membranes, and hybridized to *ADE2* and *NMD5* random-primed probes.

QAOS assay

ss DNA formation at subtelomeric regions of Chr. V-R was measured by the quantitative real-time PCR method QAOS (Booth *et al*, 2001). Briefly, cell cultures were grown in YPD at 24°C until saturation, then diluted to a final concentration of 5×10^6 cells/ml. These asynchronous cell cultures were grown for an additional 4 h at 24°C, at which point cells were centrifuged and resuspended in pre-warmed (37°C) YPD. Aliquots were then processed as described (Booth *et al*, 2001).

ChIP

ChIP experiments at the HO-generated DSB (Figure 5; Supplementary Figures S3 and S5) were performed with asynchronous cell cultures as described previously (Bianchi *et al*, 2004). ChIP experiments using the Cre–LoxP system (Figure 4) were performed as described previously (Bianchi and Shore, 2007a, b). Despite very similar overall binding patterns in the two strains in each experiment, due to variations in the overall efficiency of immuno-precipitation between different experiments, results (calculated as a percentage of input present in the immunoprecipitates) were first normalized against a value obtained by averaging the signal for the Chr. VI-R telomere within each experiment.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

Acknowledgements

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