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THE HSP82 MOLECULAR CHAPERONE CAN PROMOTE A SWITCH BETWEEN UNEXTENDABLE AND EXTENDABLE TELOMERE STATES

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Abstract

Distinct protein assemblies are nucleated at telomeric DNA to both guard the ends from damage and lengthen the DNA following replication. In yeast, Cdc13 recruits either Stn1/Ten1 to form a protective cap or the telomerase holoenzyme to extend the DNA. We have established an in vitro yeast telomere system in which Stn1/Ten1-unextendable or telomerase-extendable states can be observed. Both assemblies are Cdc13-dependent, as the Cdc13 C-terminal region supports Stn1/Ten1 interactions and the N-terminal region contains a telomerase activation function. Notably, the yeast Hsp90 chaperone Hsp82 mediates the switch between the telomere capping and extending structures by modulating the DNA binding activity of Cdc13. Taken together, our data demonstrate that the Hsp82 chaperone facilitates telomere DNA maintenance by promoting transitions between different operative complexes and by reducing the potential for binding events that would otherwise block the assembly of downstream structures.

Introduction

Telomeres are heterogeneous nucleoprotein assemblies localized at eukaryotic chromosome termini that serve both to protect the ends from damage and to extend the DNA length following replication. To accomplish these activities various proteins are nucleated at telomeric DNA to form distinct structures, including capping and extending complexes^{1,2}. While the exact composition of each assembly is not entirely understood, genetic analysis in the budding yeast *Saccharomyces cerevisiae* has revealed many critical components, including *EST2*, *STN1*, *TEN1* and *CDC13*. *EST2* encodes the telomerase reverse transcriptase subunit that is responsible for extending telomeric DNA and the Stn1 and Ten1 proteins function to cap the chromosome ends. Cdc13 promotes both DNA extension and protection—in vivo studies support a model in which Cdc13 recruits and stabilizes both Est2-extending and Stn1/Ten1-capping structures^{3–7}. Remarkably, the telomere protein system functions effectively in vivo despite a common binding specificity for single-stranded telomeric DNA by Cdc13, Stn1/Ten1 and telomerase^{4,8,9}. Why the proteins do not

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interfere with each other, how transitions between the different telomere structures are mediated and how Cdc13 serves in these seemingly opposed telomere activities (*i.e.*, capping *vs.* extending) has been unclear.

In addition to Cdc13, the yeast Hsp90 molecular chaperones Hsp82 and Hsc82 affect both capping and extending components. Hsp82 and Hsc82 are high-copy suppressors for the capping mutants *stm1-1* and *cdc13-1* yet in a wild type background elevated chaperone levels result in shortened telomeric DNA¹⁰. Neither the mechanism for the suppression nor the reduced telomere DNA length upon Hsp82-overexpression in wild type cells is understood. In contrast, the means by which Hsp82 modulates telomerase has been revealed. Human Hsp90 was the first telomerase cofactor discovered to directly influence DNA extension activity *in vitro*¹¹; work on the yeast system showed that Hsp82 promotes both telomerase DNA binding and nucleotide affinity during DNA extension¹². Hence, Hsp90 chaperones affect both capping and extending telomere components though the mechanistic contributions to the capping factors had yet to be revealed.

We sought to test directly whether the Hsp82 molecular chaperone might function with Cdc13 alone or in association with Stn1 and Ten1, by [a] establishing the functional effect of Cdc13 alone and in conjunction with Stn1/Ten1 *in vitro*, [b] demonstrating whether Hsp82 affects the different protein assemblies, and [c] determining how a switch occurs between different operative telomere-protein states.

Results

Cdc13 stimulates telomerase DNA extension activity

As a first step we determined the Cdc13 effect on telomerase DNA extension activity *in vitro*. We anticipated that Cdc13 would inhibit telomerase function by competing for the DNA substrate, as observed for the human Cdc13 ortholog Pot1¹³. To discriminate Cdc13 DNA binding-dependent effects we used DNA substrates with either 23- or 7-nucleotide 3'-overhangs in the telomerase extension assays. Prior biochemical studies indicated that Cdc13 requires a minimum of 11 single-stranded nucleotides to bind DNA¹⁴. In accordance, we found that our Cdc13 protein did not bind to DNA with a 7-base 3'-overhang but did bind to a 22-nucleotide overhang substrate (Supplementary Fig. 1). Unexpectedly, Cdc13 activated telomerase DNA extension activity independent of 3'-overhang DNA length (Fig. 1a). The DNA products below the +1 position for the 23-base substrate are likely produced by a previously described telomerase-associated endonuclease activity¹⁵.

Cdc13 enhanced the extension of the 23-base substrate to a greater extent relative to the 7-nucleotide 3'-overhang DNA (~18- *vs.* ~9-fold) suggesting that DNA binding by Cdc13 positively contributed to telomerase activity. Nonetheless, the Cdc13 activation with a 7-base substrate suggested that Cdc13 could affect telomerase independent of direct DNA binding. Notably, we found that Cdc13 assembled with telomerase or potentially some other RNase-sensitive component of the extract on 7- or 22-base 3'-overhang substrates but that the 7-base DNA structure was dependent upon telomerase-DNA binding activity, which suggests that Cdc13 tethers to the DNA-bound enzyme (Fig. 1b). While stimulation by Cdc13 contrasts with the inhibitory effect of Pot1, it should be noted that Pot1 activates

telomerase in conjunction with the telomeric protein Tpp116. Hence, Cdc13 apparently functions in a manner comparable to the combined effects of Pot1 and Tpp1.

The Cdc13 amino-terminus has a telomerase activation function

To better understand how Cdc13 regulates telomerase activity we produced and characterized a series of Cdc13 protein derivatives that included full-length (FL), Cdc13-2 point mutant (2), DNA binding domain (DBD), amino-terminus through the DBD (N) and DBD through the carboxyl-terminus (C) (Fig. 2); all derivatives displayed high-affinity DNA binding activity (data not shown). The FL, Cdc13-2 and the N-fragment activated telomerase DNA extension activity on either DNA substrate. Hence, in addition to its suggested telomere recruitment function⁶, the Cdc13 N-terminus has a telomerase activation surface. In contrast, the C or DBD derivatives inhibited extension of the 23-base substrate but did not affect basal extension of the 7-base 3'-overhang DNA (Fig. 2).

The ability of Cdc13-2 to stimulate telomerase indicates that the up-regulation does not involve interactions with the holoenzyme component Est1, as the Cdc13-2 mutation disrupts Cdc13-Est1 interactions, and likely is distinct from the Cdc13 telomere recruitment function⁷. We believe that the observed telomerase activation potential likely occurs downstream from nucleation of telomerase to a telomere in vivo. The positive effect of the N-domain on telomerase extension activity might account, in part, for the increased telomere DNA length in *cdc13-5* yeast⁶. Assuming telomerase remains telomere engaged following a single Cdc13-mediated recruitment event, then an additional Cdc13 function would be required to not only account for the hyper elongation of the DNA in the *cdc13-5* background but to also counter the DNA attrition that would be anticipated by the loss of the carboxyl-terminal Stn1 interaction site¹⁸.

In contrast to activation, telomerase inhibition by the C and DBD fragments was dependent upon the length of the single-stranded DNA, which suggests the decline in activity likely results from competitive binding to the DNA substrate. If these suppositions are correct then proper telomere regulation in vivo would require a mechanism to prevent Cdc13 from competing with telomerase for DNA binding without interfering with Cdc13-regulatory capabilities.

Cdc13, Stn1 and Ten1 cooperate to form an unextendable telomere DNA complex

In addition to Cdc13, Stn1 and Ten1 serve in telomere capping in vivo¹. To determine whether FL-Cdc13 can function with Stn1 and Ten1 we purified the proteins (Supplementary Fig. 2a) and tested the effects of the proteins on telomerase activity. In the presence of Cdc13 we observed a striking reduction in DNA extension with increasing Stn1/Ten1 levels using the 23-base 3'-overhang DNA but not the 7-base substrate (Fig. 3a and Supplementary Fig. 2b). In the absence of Cdc13, Stn1/Ten1 had no apparent effect on DNA extension (Fig. 3a). Importantly, the point mutant Cdc13-2, which does not interact with Stn1 in vivo⁶, fails to repress telomerase activity with Stn1/Ten1 in vitro (Fig. 3b) indicating that the Stn1/Ten1 interaction is specific for wild type Cdc13. Our in vitro data support recent in vivo work suggesting Cdc13 and Stn1 function to block telomerase action at a telomere¹⁷.

The negative effect of the DBD and C-domain fragments on telomerase activity complicated our ability to assess whether these proteins can form an Stn1/Ten1-cap structure since DNA extension activity is suppressed in the absence of Supplementary Stn1/Ten1. To determine which Cdc13 domain assembles with Stn1/Ten1 on DNA we performed fluorescence anisotropy DNA binding experiments. We found that both FL and the C-domain associated with Stn1/Ten1 on DNA in vitro (Fig. 3c), which agrees with prior genetic data demonstrating that the C-terminus can interact with Stn1 to protect telomere ends in vivo¹⁸. In contrast, the N-terminus and Cdc13-2 fail to assemble with Stn1/Ten1 on DNA in vitro (Fig. 3c). Comparable results were observed using the electro-mobility shift assay (Supplementary Fig. 2c). Hence, Cdc13 has three distinct domains—an amino-terminal telomerase activation region, a central DNA binding domain and a carboxyl-terminal Stn1/Ten1 interaction site.

Hsp82 converts the telomere unextendable state to an extendable form

The ability of the DBD and C-domain to interfere with DNA extension indicates that Cdc13 DNA binding activity is sufficient to inhibit telomerase, yet full-length Cdc13 only activates, unless Stn1/Ten1 is also supplemented. To account for these results we believed that a telomerase cofactor was present in the reactions, preventing Cdc13 from interfering with telomerase but still permitting DNA-bound telomerase to associate with Cdc13. Given the established Hsp82 roles with capping and extending telomere components, we tested whether Hsp82 might affect Cdc13. As an initial test Hsp82 was titrated into DNA extension reactions containing a Cdc13/Stn1/Ten1-capped telomeric DNA substrate (Fig. 4a). In the absence of Supplementary Hsp82, Cdc13/Stn1/Ten1 suppressed the DNA extension activity below basal levels (Fig. 4a lanes 1 & 3). However, in an Hsp82-dependent manner the extension activity increased ~15-fold, which indicates that Hsp82 is sufficient to switch the Cdc13/Stn1/Ten1-capping structure into a Cdc13/telomerase-extending complex (Fig. 4a).

To investigate how Hsp82 affects the protein-DNA complexes, we determined the impact of Hsp82 on the DNA binding activities of the Cdc13 derivatives. We found that Hsp82 dissociated FL Cdc13 and the N-domain from DNA but the chaperone did not extensively affect DNA binding by the DBD or C-terminal fragment (Fig. 4b). Hence, Hsp82 modulates Cdc13 through interactions with the N-terminus. In addition to direct DNA binding, we found that Cdc13 and the DBD fragment could protect single-stranded DNA from nuclease digestion (Supplementary Fig. 3). However, in the presence of Hsp82 the full-length Cdc13 no longer protected the DNA. Based on the anisotropy data, Hsp82 displayed a higher affinity for the N-fragment ($K_d \sim 35$ nM) relative to FL ($K_d \sim 325$ nM) suggesting contacts between the amino- and carboxyl-domains of Cdc13 impact modulation by Hsp82. In contrast to Hsp82, Sba1 had no apparent effect on Cdc13 DNA binding despite the ability of Sba1 to dissociate telomerase from DNA (Fig. 4b)¹⁹.

Notably, we found that Hsp82 actively prevented Cdc13 DNA binding, as Cdc13 binding activity was restored upon Hsp82 inhibition (Fig. 5a). Furthermore, treatment of telomerase extract with an Hsp82 small-molecule inhibitor (radicol) resulted in Cdc13-mediated suppression of telomerase activity using the 23-base substrate rather than activation (Fig. 5b). Thus, Hsp82 specifically converts the Cdc13-capping structure into a Cdc13/

telomerase-extending complex by interfering with DNA binding by Cdc13 but not with its ability to associate with DNA-bound telomerase. In addition, we found that alternate stepwise addition of Hsp82 and Stn1/Ten1 revealed a competitive equilibrium effect on the DNA binding activity in vitro (Fig. 5c). Thus, Hsp82 can dissociate the Cdc13/Stn1/Ten1 bound complex yet the capacity of additional Stn1/Ten1 to counter the Hsp82 dissociation activity provides a mechanism to reestablish a Cdc13 DNA-bound state, which has important implications for Cdc13-mediated DNA structures.

Hsp82 modulates Cdc13 telomere occupancy and telomere activities in vivo

Given the impact of Hsp82 on Cdc13 in vitro, one might predict that Hsp82 overexpression would have a marked effect on telomere DNA length in vivo. Yet, our studies and prior work indicate that ectopic Hsp82 overexpression results in an ~50% reduction in telomere DNA length (Supplementary Fig. 4a)¹⁰. Though the change in DNA length does correlate with the relative increase in Hsp82 protein amounts (~2-fold; Supplementary Fig. 4a), the modest telomere effect might also occur if Hsp82 is affecting competing telomere functions. For example, an increase in cellular Hsp82 might lower Cdc13-mediated DNA end protection by limiting formation of capping complexes; however, a reduction in Cdc13 DNA-binding would promote telomerase-DNA interactions by elevating telomerase accessibility to the extreme 3' DNA end.

To test this model we examined the Hsp82 effect on the cell cycle telomere occupancies by Cdc13 and telomerase and the Hsp82 impact on telomere capping and extending activities. We found that telomere association by Cdc13 declined upon Hsp82 overexpression throughout the cell cycle except during late S phase (Fig. 6a). Thus, we observed the occupancy pattern that would be predicted based upon our in vitro data. Cdc13 telomere interactions are reduced during cell cycle phases associated with capping events but are unaffected at late S phase when Cdc13 would activate telomerase by tethering to the DNA-bound enzyme. To determine if the declined Cdc13 telomere occupancy affects DNA end protection we checked whether the relative levels of single-stranded TG₁₋₃ DNA fluctuated with Hsp82 expression using an in-gel native Southern blot assay^{20,21}. As might be anticipated, we observed a TG₁₋₃ signal increase in yeast overexpressing Hsp82 (Supplementary Fig. 4b), which suggests DNA end protection declines with increased Hsp82 protein in vivo.

In contrast to Cdc13, we found that Est2 telomere interactions did not change appreciably with Hsp82 expression except for a modest but reproducible (albeit statistically insignificant) increase in late S phase (Fig. 6b). Minimally, the data show that additional Hsp82 does not interfere with the ability of Cdc13 to efficiently recruit telomerase to telomeres. We suspect that the mild rise in Est2-telomere association results from an increase in direct DNA binding by telomerase (*i.e.*, Est2-DNA crosslinking efficiency has increased as more Est2 is in direct contact with the DNA). If elevated Hsp82 levels do facilitate telomerase access to the telomeric DNA then there might be an increase in telomere DNA extension rates since telomerase would now be directly engaged with the DNA.

To investigate telomere DNA lengthening activity, we exploited an established, engineered yeast strain that permits controlled, rapid reduction in telomere DNA length and assessment of telomerase DNA extension rates *in vivo*²². In brief, the left arm terminus of chromosome VII contains an internal telomere tract that is flanked by Flp1-recognition target sites, the tract is specifically removed upon transient Flp1 expression and DNA elongation can be monitored in subsequent cell divisions. To limit the impact of Hsp82 effects on capping events, Hsp82 overexpression was induced in conjunction with Flp1 while the yeast were under α -factor cell cycle arrest. This permitted a focus on the DNA extension levels achieved during the first cell cycle after the Flp1-mediated telomeric DNA reduction. In the presence of elevated Hsp82 protein levels, we found that the extension length was faster (~5-fold) relative to yeast with endogenous Hsp82 levels (Fig. 6c). While there are a number of possibilities for the increased DNA lengthening including direct effects on telomerase activity by Hsp82¹², we suggest that the change also involves modulation of Cdc13 DNA binding by Hsp82. In brief, the telomerase-mediated DNA extension increases since Hsp82 inhibits assembly of the unextendable telomere structure (*i.e.*, reduces steric interference by Cdc13) without disrupting positive Cdc13 functions (*i.e.*, telomere recruitment and telomerase activation). Taken together, our data suggest that steady-state telomere DNA length is reduced upon Hsp82 overexpression due to declined capping activities. However, the magnitude of the shortening is limited since telomerase accessibility to the telomeric DNA has increased and therefore telomerase-mediated DNA extension is enhanced, which counters the impact of the declined telomere capping.

Discussion

The presented work provides a potential resolution to the apparent conundrum of how proteins with identical binding specificities coordinately function at a single site. Often DNA binding proteins form long-lived complexes with target DNAs *in vitro* that would interfere with the assembly of other structures and telomere-protein assemblies are no exception². For example, a stable Cdc13-telomere association at the extreme 3' DNA end would block telomerase DNA binding yet Cdc13 binding at the extreme termini likely is required to protect the DNA from degradation *in vivo*. Our data supports a model in which Hsp82 frees the DNA end of competing binding proteins without interfering with critical regulatory events.

In our model, telomerase recruitment to a telomere initiates through transient contacts between Cdc13 and the holoenzyme (Fig. 7). To permit proper telomerase DNA association Hsp82 displaces proteins bound at the extreme 3' DNA end including Cdc13. Recent genetic studies highlight the potential importance of maintaining a dynamic telomere environment, as the peak of telomere association by Cdc13, Stn1 and Est1 all occur in S-phase^{17,23}. Presumably, both capping (Stn1) and extending (Est1) proteins are telomere recruited each S-phase since not all telomeres are extended each cell cycle²⁴. Hence, depending upon the DNA length of a particular telomere a molecular choice is made to either permit or preclude telomerase function (*i.e.*, build an Est1-extending or Stn1-capping complex). If Hsp82 maintains Cdc13 in a dynamic DNA binding cycle, then any modifications (*e.g.*, phosphorylation) meant to guide a telomere to a distinct operative phase would be immediately incorporated.

Recent genetic studies suggest post-translational modifications mediated by the Tel1 (ATM homolog) and Cdk1 kinases are critical for proper telomere DNA maintenance^{25,26}. For example, Tel1 is recruited to critically short telomeric DNA tracts, which are preferentially elongated, and Cdc13 has been shown to be a Tel1 target^{25,27}. In addition, Cdk1 phosphorylates Cdc13 and the modification appears to influence the preference between Stn1- and Est1-containing Cdc13 nucleated telomere complexes²⁶. Hence, depending upon the Cdc13 phosphorylation-state, telomeric DNA would be in a Cdc13-Stn1/Ten1 unextendable state or a Cdc13-Est1/telomerase extendable form. Dynamic Cdc13 action, mediated by Hsp82, would enable the telomere system to rapidly transition between the different structures as needed.

In addition to protein dynamics, our studies reveal a Cdc13 telomerase activation function, which may account for the telomere lengthening phenotypes for several *cdc13* mutations^{5,6}. Of note, the Cdc13 telomerase stimulation activity that we observe is in stark contrast to a recent report showing telomerase inhibition by full-length Cdc13²⁸. Currently, the reason for the different effect of full-length Cdc13 on telomerase activity is unclear. We did find that the Cdc13 activation function was lost following a brief (5 min) incubation at 42°C while DNA binding activity was unaffected (data not shown), which suggests that the conformation of the activation surface is labile. Perhaps by expressing full-length Cdc13 in a specialized bacterial strain (*i.e.*, streptomycin-resistant strain), which was critical for obtaining soluble recombinant protein²⁹, the activation surface was provided an opportunity to properly fold. Opposed to the data on full-length Cdc13, both studies did observe telomerase inhibition by the Cdc13 DNA binding domain fragment and the DBD displayed no apparent difference when expressed in either classic or streptomycin-resistant bacterial cells²⁹.

Nevertheless, prior genetic data suggests a role for Cdc13 with telomerase that is downstream of telomere nucleation. For example, the *cdc13-4* yeast have short but stable telomeric DNA despite an apparent capacity of Cdc13-4 to recruit telomerase to telomeres and protect the DNA from degradation³⁰. While the effected residue (P235S) occurs within the amino-terminal domain, the mechanism for the telomere defect was not identified. However, the *in vivo* phenotype is consistent with a post-recruitment function for Cdc13³⁰. Intriguingly, the Cdc13-109 and Cdc13-231 mutants lead to over-elongated telomeric DNA despite a decline in DNA binding activity⁵. If the sole positive function of Cdc13 was to recruit telomerase to a telomere, then a decline in DNA binding should result in shorter not longer telomeric DNA. Additionally, the hyper-elongation of telomeric DNA in the *cdc13-5* yeast is consistent with a post-recruitment role for Cdc13. The Cdc13-5 protein (N-domain) consists of the amino-terminus and DNA binding domain⁶. In the absence of the Cdc13 carboxyl-terminal Stn1 interaction site the telomeric DNA should be vulnerable to nuclease attack. Yet, the telomeric DNA is not degraded in the *cdc13-5* yeast but rather is over-elongated⁶. We suggest that, in addition to recruiting telomerase, Cdc13-5 activates telomerase to not only hyper-extend the DNA but also to counter any potential DNA degradation. Of note, the stimulatory function is downstream from Cdc13-mediated recruitment since the effect is abrogated when combined with the Cdc13 point mutation *cdc13-2* (*i.e.*, *cdc13-2,5*)⁶. Taken together, established genetic data support our contention

that Cdc13 modulates the DNA-bound enzyme. We suggest that telomerase cofactors, including Cdc13, likely reengage the telomere protein assembly by tethering to the DNA-bound telomerase enzyme.

A chaperone-mediated protein dynamics model has been previously proposed for transcription pathways³¹. In brief, molecular chaperones promote a dynamic action for transcription factors that is necessary to permit rapid functional recruitment of multiple coactivating complexes to a gene promoter. An important distinction, however, is the impact of the Hsp90 chaperone: Hsp90 promotes DNA binding by transcription factors but facilitates dissociation of Cdc13-DNA complexes. Perhaps the dual role for Hsp82 at telomeres provides an explanation for the difference. By exploiting the telomerase-associated cofactor Hsp82 to both support telomerase function and to clear the telomeric DNA of competing proteins, an elegant means to ensure telomeric DNA extension within the short working period allotted telomerase at the end of S phase is provided.

Traditionally Hsp90 has been viewed as a cytoplasmic molecular chaperone required for the late folding stages of signaling molecules³². However, recent studies including high-throughput screens have identified a broad-range of potential nuclear client proteins². Given the impact of Hsp90/Hsp82 both on telomeric and transcriptional targets, there appears to be a general cellular role for Hsp90 chaperones in controlling protein-DNA dynamics. In brief, multi-step pathways, including the telomere system, move forward through high affinity interactions between the low abundant proteins unique to that system (*e.g.*, Cdc13, telomerase, Stn1) while proper structure composition (*i.e.*, competitive interactions) along with efficient transitions between the different assemblies are mediated by transient, low affinity interactions with the highly abundant molecular chaperones.

Methods

Protein purification

We expressed the Cdc13 protein derivatives (pET28) and Stn1 (pETDuet1) protein as amino-terminal (His)₆ fusions using T7 expression vectors in streptomycin resistant *Escherichia coli* cells CH184. Protein expression in the CH184 background alleviated solubility problems associated with Cdc13 and Stn1/Ten1. The Ten1 (pETDuet1) protein was coexpressed and copurified with the His-tagged Stn1. The Hsp82 was expressed as an amino-terminal (His)₆ fusion using a T7 expression vector (pET28) in codon optimized Rosetta *Escherichia coli* cells (Novagen Inc.). For the telomeric proteins the cells were grown at 18°C, LB media lacking streptomycin was seeded to an O.D.₅₉₅ = 0.2 with transformants from an overnight culture grown in LB supplemented with streptomycin (0.2 mg mL⁻¹), the cells were grown to an O.D.₅₉₅ = 0.8, protein expression was induced with IPTG and the cells were incubated for an additional 4 h. For Hsp82 expression the cells were grown comparably except streptomycin was not used in the overnight seed culture and cells were incubated at 24°C. After IPTG induction the cultures were clarified by centrifugation, resuspended in ice-cold 1x Talon binding buffer, and snap frozen; NP40 (1% v/v final) was added to the Cdc13 preparations. The proteins were purified using metal affinity chromatography (Talon resin) as per manufacture's instructions (Clontech Inc.). The telomeric proteins were further resolved over MonoQ anion exchange resin and a

Superdex-200 size exclusion column (GE Inc.). The Hsp82 was purified as previously described¹². The identities of the purified proteins were confirmed by mass spectrometry and/or western blot analysis.

Telomerase DNA extension assays

Telomerase extracts were prepared using wild type (YPH499) *Saccharomyces cerevisiae* and the telomerase DNA extension reactions were performed as described¹⁹. In brief, the DNA extension reactions used MonoQ telomerase extract in extension buffer (50 mM Tris pH 8.0, 1.0 mM MgCl₂, 1.0 mM spermidine, 1.0 mM DTT, 0.5% (v/v) glycerol, 50 μM dTTP, 20 mCi [α -³²P] dGTP) with 2.0 pmol telomeric DNA substrate immobilized on paramagnetic beads and supplemented with the indicated proteins. Following cleavage of the DNA substrates with *Eco*RI the released DNA fragments were magnetically separated from the beads, an end-labeled precipitation/loading control primer was added, the DNAs were subjected to ethanol precipitation, the products were reconstituted in formamide/NaOH loading buffer, subjected to denaturing gel electrophoresis and visualized using a Molecular Dynamics PhosphoImager.

Fluorescence anisotropy assay

The anisotropy reactions were performed in 25 μL volumes containing TMG-30 supplemented with a fluorescein-labeled telomere oligonucleotide (12.5 nM; fl-GTGGGTGTGTGTGTGGGGTGGTGTGTGTGG). To form the hybrid single-/double-stranded DNA substrates shorter complimentary oligonucleotides were annealed to the 5' end of the telomere primer—7-base (CACCACCCACACACACCCAC), 15-base (CACACACACCCAC) or 22-base (ACACCCAC) 3'-overhang. The reactions were incubated 5 min at 25°C before determining the anisotropy values using an Ultra Evolution plate reader (Tecan Inc.). RNase sensitivity of telomerase DNA binding was determined by preincubating aliquots of the MonoQ extracts with 10 mg of RNase A for 10 min at room temperature¹⁹. All anisotropy data represent average values (mean \pm SD) from 5 independent assays.

Chromatin immunoprecipitation assay

ChIP analysis was essentially performed as described¹⁹. For the cell cycle experiments yeast were arrested in late G₁ with α -factor (10 μg mL⁻¹), following a 2 h incubation the cultures were clarified by centrifugation and resuspended in rich media lacking α -factor to allow synchronous progression through the cell cycle at 30°C. Aliquots were removed to monitor cell cycle synchronization by FACS and budding assay or treated with formaldehyde (1% v/v final). To determine the relative telomere occupancy by Cdc13 or Est2 transformants (pRS425-*GPD* or pRS425-*GPD-HSP82*) of either the *CDC13*-MYC9x or *EST2*-MYC9x yeast²³ were utilized. The fold-enrichments were determined by normalizing the ratio of the specific/nonspecific signal (*e.g.*, α -Myc ChIP threshold cycle (C_t values)/normal mouse IgG ChIP C_t value) at the telomeric DNA targets to the ratio of the specific/nonspecific signal produced by amplifying a non-telomeric region of the genome (YJL052W).

Telomere DNA length analysis

Genomic DNA was prepared from yeast (YPH499) transformants (pRS425-*GPD* or pRS425-*GPD-HSP82*) after the indicated passages (~8–10 generations per passage). The DNA was digested overnight with *Xho*I, resolved on a 25 cm 1% (w/v) agarose gel for 20–24 h at 40 V and the DNA was transferred to Immobilon-Ny⁺ membrane (Millipore Inc.). The telomeric DNA was identified following hybridization in Ekono buffer (ISC BioExpress Inc.) supplemented with a radiolabeled telomeric oligonucleotide ((TGTGGGT)₄). As a loading and migration control we used an established probe that recognizes a 1,621 bp fragment of chromosome IV33. The probe was generated by PCR amplification of genomic DNA in the presence of α -dGTP using specific oligonucleotides (GTACCTCGGTTTAGTTAAGCG and CTCATTCGAATCCATACGACC). Following appropriate washes using SSC/SDS buffers the telomeric and control DNA was visualized using a PhosphorImager (Molecular Dynamics Inc.).

Telomere DNA extension assay

The telomere elongation assay was performed as described²². Three representative data sets are shown in Supplementary Fig. 5.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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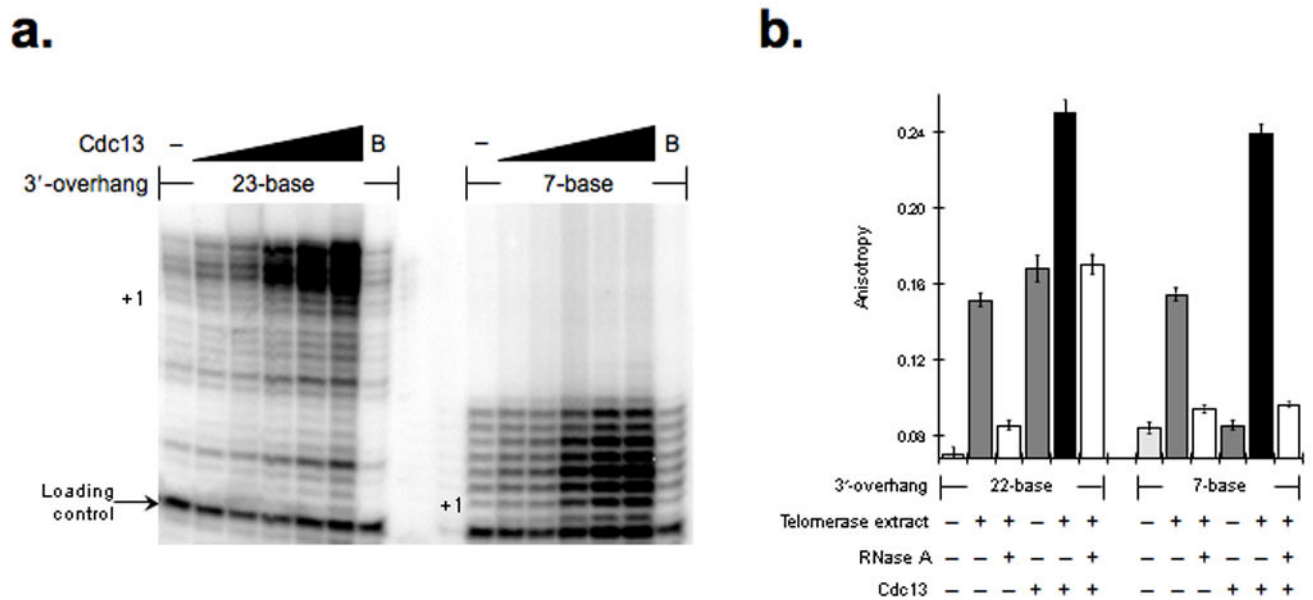


Figure 1.

Cdc13 stimulates telomerase DNA extension activity independent of single-stranded 3'-overhang DNA length. **(a)** The Cdc13 effect on telomerase-mediated DNA extension was determined using DNA substrates with either 23- or 7-nucleotide 3'-overhangs and a Cdc13 protein titration (50, 100, 250, 500 and 1000 nM), as marked. To control for possible non-specific protein affects the impact of BSA (B) (1000 nM) addition was tested. All extension reactions were supplemented with a loading control primer (arrow) prior to precipitation and electrophoretic resolution and the +1 position for each DNA substrates is marked. **(b)** Cdc13 can tether to DNA-bound telomerase. Fluorescence anisotropy with a fluorescein-labeled telomeric oligonucleotide was used to detect DNA association. The binding activities of Cdc13 (250 nM) or telomerase alone on 22- and 7-base DNA substrates were examined and the ability of Cdc13 to join DNA-bound telomerase was determined by adding purified Cdc13 (250 nM) to telomerase-DNA complexes using either the 22- and 7-base DNA substrates, as indicated. To demonstrate telomerase-dependent DNA binding RNaseA treatment was included. The quantified data represent average values (mean \pm s.d.) from 5 independent assays.

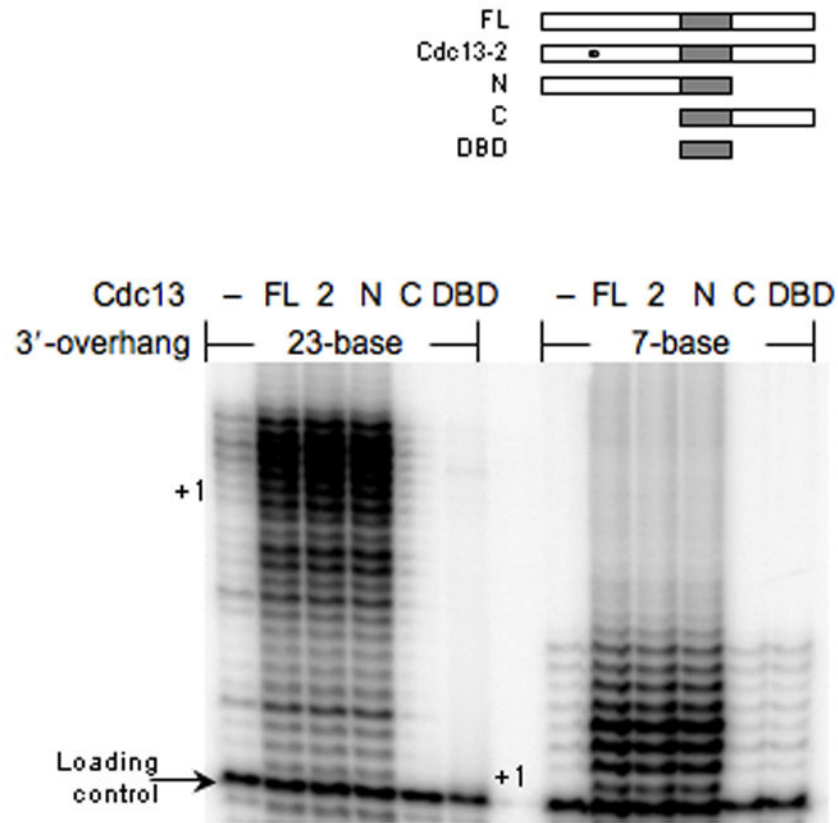
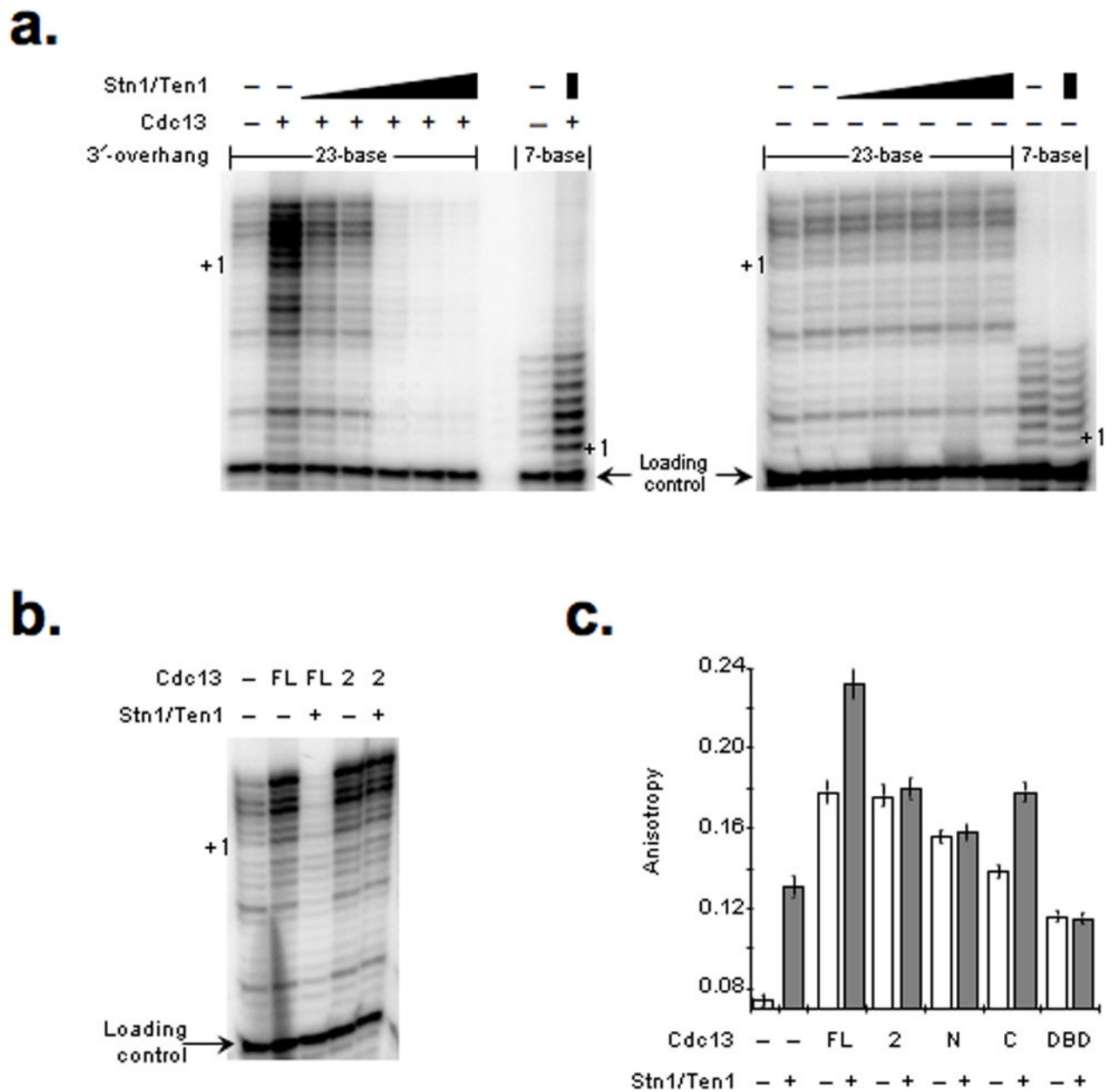


Figure 2.

The amino-terminal domain of Cdc13 has a telomerase-activation function. The effect of full-length (FL) Cdc13, Cdc13-2 (2), amino-terminus (N), carboxyl-terminus (C) or DNA binding domain (DBD) proteins (250 nM) on telomerase DNA extension using 7- or 23-base 3'-overhang DNA substrates was determined. All extension reactions were supplemented with a loading control primer (arrow) prior to precipitation and electrophoretic resolution and the +1 position for each DNA substrates is marked.

**Figure 3.**

Stn1 and Ten1 cooperate with Cdc13 to form an unextendable telomere protein-DNA complex *in vitro*. (a) The effect of an Stn1/Ten1 protein titration (50, 100, 250, 500 and 1000 nM) on telomerase DNA extension in the presence or absence of Cdc13 (250 nM) was determined using 23- or 7-base 3'-overhang DNAs. For comparison, the activity of unsupplemented and Cdc13 supplemented telomerase extract is shown. (b) Cdc13-2 does not form an unextendable DNA complex with Stn1/Ten1. Telomerase DNA extension reactions were performed using a 23-nucleotide 3'-overhang substrate and the reactions were supplemented with Cdc13 (FL) (250 nM) or Cdc13-2 (2) (250 nM) with or without Stn1/Ten1 (250 nM), as marked. All extension reactions were supplemented with a loading

control primer (arrow) prior to precipitation and electrophoretic resolution and the +1 position for each DNA substrates is indicated. (c) The carboxyl-terminus of Cdc13 harbors an Stn1/Ten1 interaction surface. Fluorescence anisotropy with a fluorescein-labeled 15-base 3'-overhang telomeric oligonucleotide was used to detect DNA association; the 15-base DNA section provides a single Cdc13 binding site. The anisotropy was measured for each Cdc13 derivative (100 nM) alone and in the presence of Stn1/Ten1 (100 nM). The quantified data represent average values (mean \pm s.d.) from 5 independent assays.

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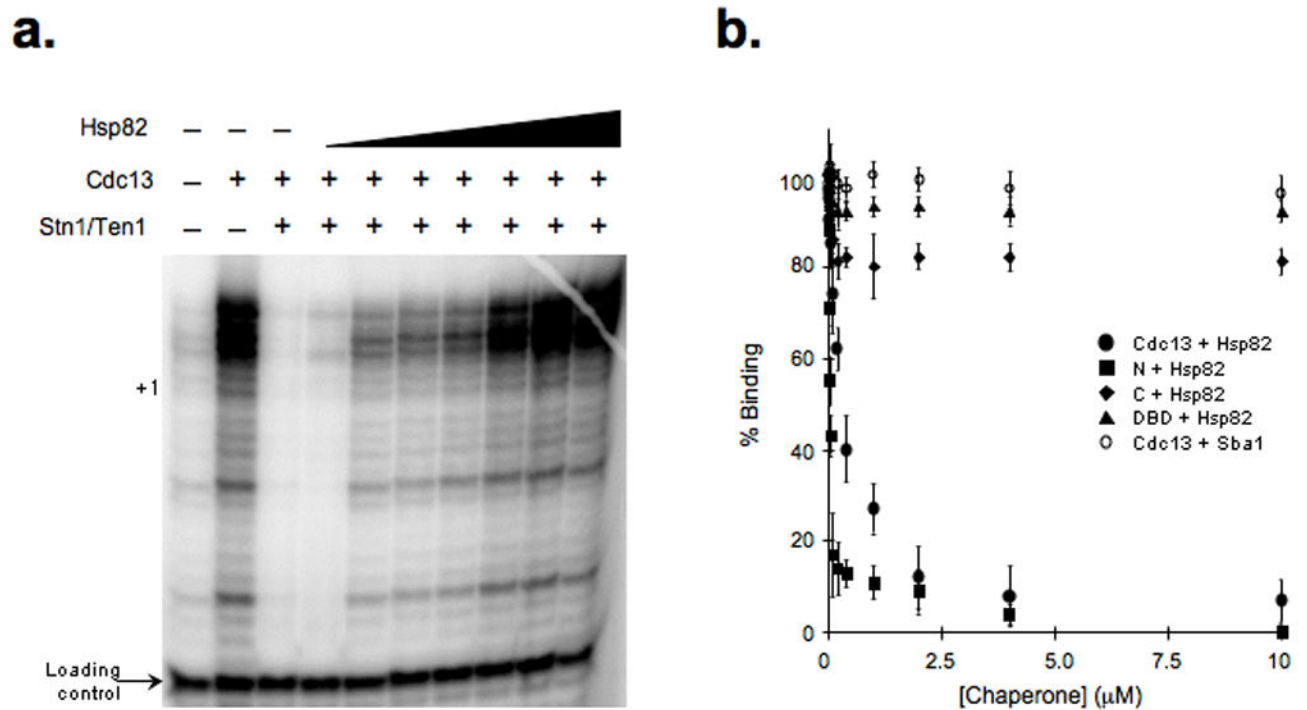
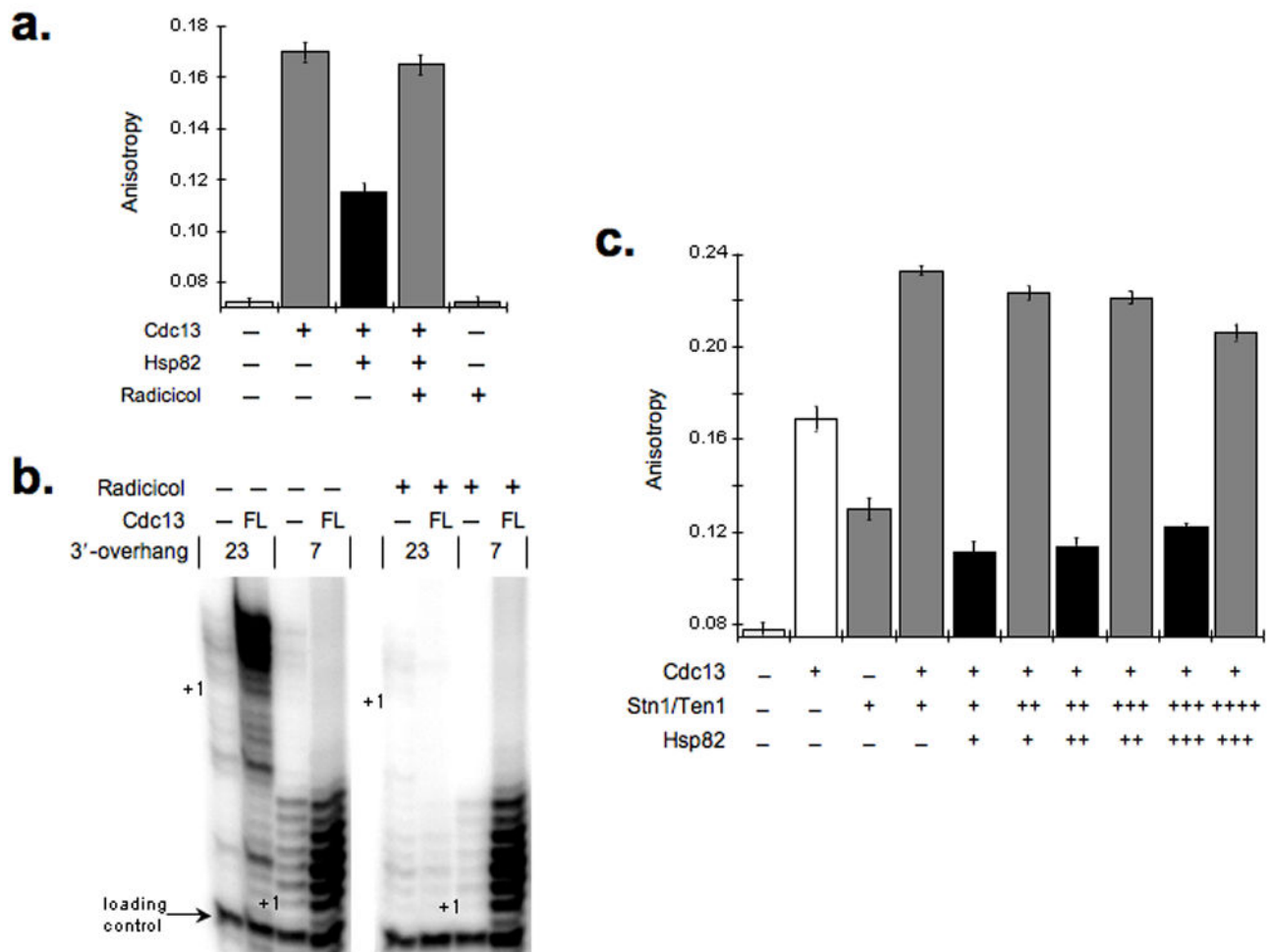
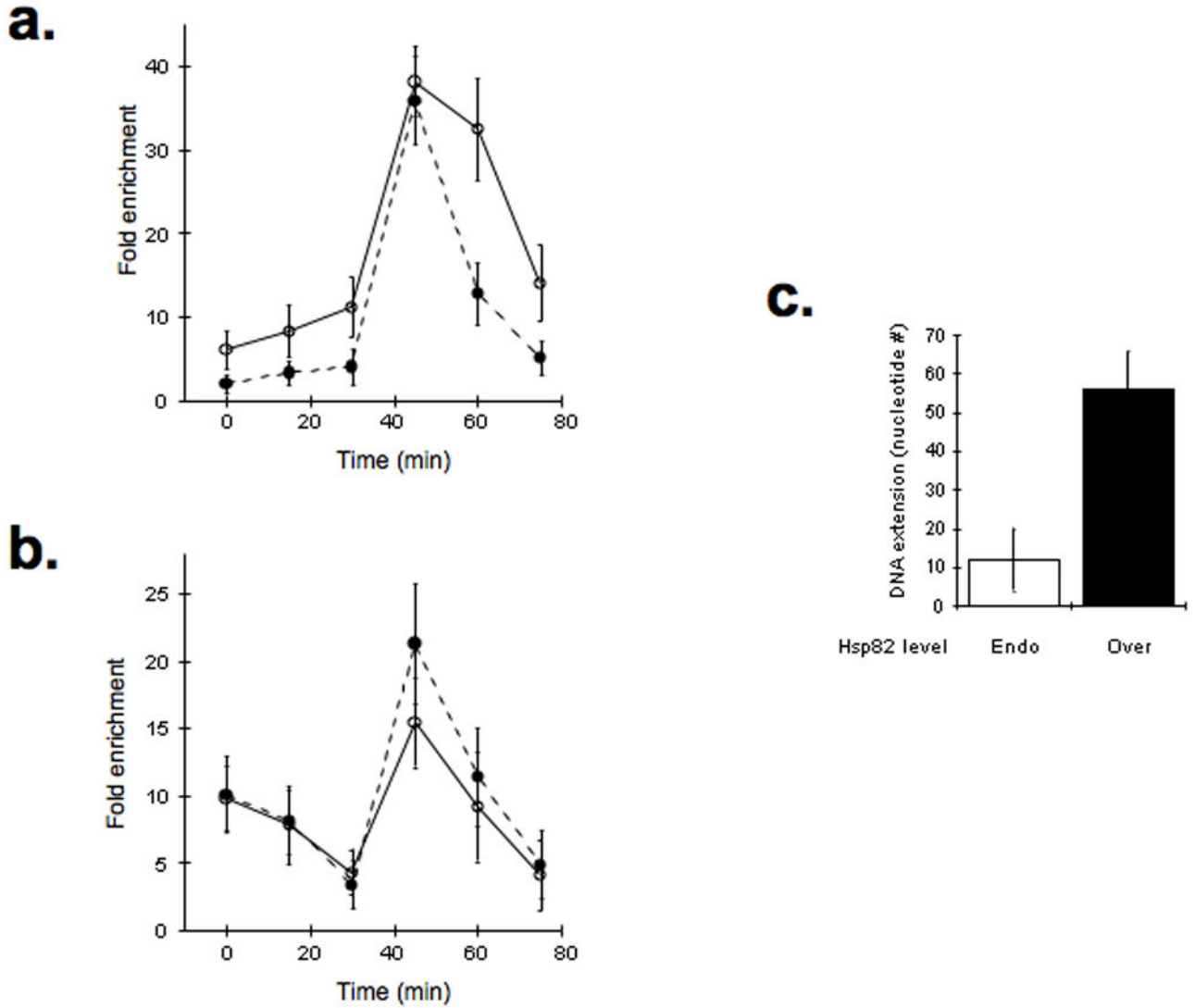


Figure 4.

Hsp82 promotes conversion of the Cdc13-capping structure into a Cdc13-extending complex by dissociating Cdc13 from DNA. **(a)** The ability of Hsp82 to convert an unextendable Cdc13/Stn1/Ten1-capped DNA complex into a telomerase accessible DNA structure was determined using a 23-base 3'-overhang DNA substrate, telomerase extract and an Hsp82 protein titration (0.25, 0.5, 1.0, 2.5, 5.0, 10.0 and 20.0 μM). The cellular Hsp82 concentration is 17 μM ³⁴. **(b)** Hsp82 dissociates amino-terminal Cdc13 protein derivatives from DNA. Cdc13 DNA binding was monitored by fluorescence anisotropy. The impact of Hsp82 on the binding activities of FL, C, N or DBD Cdc13 protein derivatives (100 nM) was determined along with the effect of Sba1 on FL, as indicated.

**Figure 5.**

Hsp82-mediated Cdc13-DNA dissociation is an active process. **(a)** Addition of the Hsp82 small-molecule inhibitor Radicicol reestablishes Cdc13 DNA binding activity. Anisotropy values for a fluorescein-telomeric oligonucleotide upon the consecutive addition of Cdc13 (100 nM) then Hsp82 (1.0 μ M) and finally Radicicol (20 μ M) is shown. **(b)** Wild type Cdc13 impedes telomerase activity on a 23-base DNA substrate upon small molecule inhibition of Hsp82. Telomerase DNA extension reactions were performed using 23- or 7-nucleotide 3'-overhang substrates. The reactions were supplemented with Cdc13 (250 nM) alone or with Radicicol (20 μ M). As expected, loss of Hsp82 function resulted in a declined telomerase DNA extension activity¹². All extension reactions were supplemented with a loading control primer (arrow) prior to precipitation and electrophoretic resolution and the +1 position for each DNA substrates is marked. **(c)** Hsp82 and Stn1/Ten1 can drive a competitive equilibrium for Cdc13 DNA binding. Aliquots of Stn1/Ten1 (250 nM) and Hsp82 (1 μ M) were added sequentially to a reaction mix of fluorescein-telomeric oligonucleotide and Cdc13 (250 nM). The anisotropy values were measured after each addition. The quantified data represent average values (mean \pm s.d.) from 5 independent assays.

**Figure 6.**

Hsp82 affects telomere events in vivo. Hsp82 affects Cdc13-telomere association in vivo. The cell cycle-dependent telomere occupancy by Cdc13 (a) and the telomerase reverse transcriptase subunit Est2 (b) were determined using the chromatin immunoprecipitation (ChIP) assay in either *CDC13-9xMYC* or *EST2-9xMYC*, respectively. Yeast transformants with endogenous (open symbols) or overexpressed (closed symbols) Hsp82 levels were synchronized with α -factor. (c) Telomeric DNA extension is HSP82-dependent. Elongation of inducibly truncated telomeric DNA was monitored in yeast with endogenous (Endo) or overexpressed (Over) Hsp82, as indicated. The quantified data represent average values (mean \pm s.d.) from 5 independent assays. The average number of nucleotides added to the shorten-telomeric DNA was determined using an established protocol²².

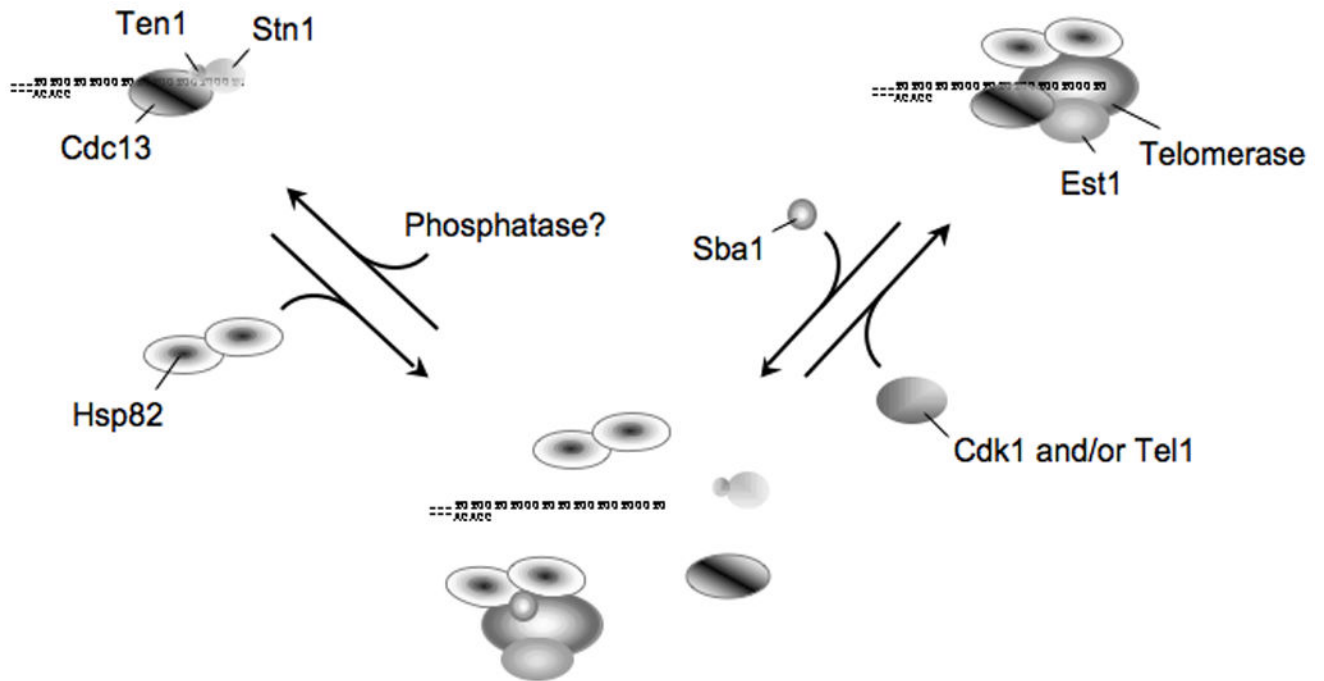


Figure 7.

Hsp82 mediates telomere-protein dynamics. Cdc13 recruits select proteins to telomeric DNA including Stn1/Ten1 and telomerase/Est1. As the independent structures form stable and long-lived interactions with telomeric DNA, transitions between the distinct complexes requires molecular chaperone-mediated disassembly^{2,19}. Specific post-translational modifications would then guide the assembly of the requisite structure, which minimally would vary with the phase of the cell cycle. For example, phosphorylation of Cdc13 by Cdk1 and/or Tel1 would promote telomerase nucleation and DNA extension events^{25,26}.