DOI: 10.1534/genetics.109.108894

TEN1 Is Essential for *CDC13*-Mediated Telomere Capping

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Manuscript received August 22, 2009
Accepted for publication September 6, 2009

ABSTRACT

Telomere binding proteins protect chromosome ends from degradation and mask chromosome termini from checkpoint surveillance. In *Saccharomyces cerevisiae*, Cdc13 binds single-stranded G-rich telomere repeats, maintaining telomere integrity and length. Two additional proteins, Ten1 and Stn1, interact with Cdc13 but their contributions to telomere integrity are not well defined. Ten1 is known to prevent accumulation of aberrant single-stranded telomere DNA; whether this results from defective end protection or defective telomere replication is unclear. Here we report our analysis of a new group of *ten1* temperature-sensitive (ts) mutants. At permissive temperatures, *ten1-ts* strains display greatly elongated telomeres. After shift to nonpermissive conditions, however, *ten1-ts* mutants accumulate extensive telomeric single-stranded DNA. Cdk1 activity is required to generate these single-stranded regions, and deleting the *EXO1* nuclease partially suppresses *ten1-ts* growth defects. This is similar to *cdc13-1* mutants, suggesting *ten1-ts* strains are defective for end protection. Moreover, like Cdc13, our analysis reveals Ten1 promotes *de novo* telomere addition. Interestingly, in *ten1-ts* strains at high temperatures, telomeric single-stranded DNA and Rad52-YFP repair foci are strongly induced despite Cdc13 remaining associated with telomeres, revealing Cdc13 telomere binding is not sufficient for end protection. Finally, unlike *cdc13-1* mutants, *ten1-ts* strains display strong synthetic interactions with mutations in the *POL*α complex. These results emphasize that Cdc13 relies on Ten1 to execute its essential function, but leave open the possibility that Ten1 has a Cdc13-independent role in DNA replication.

GENOME stability is critically dependent upon functional telomeres. DNA ends that lack telomeres, or that have dysfunctional telomeres, are metabolized by DNA repair processes; without an appropriate repair template, such chromosome ends can be resected or joined inappropriately with other chromosome ends. Thus, genomic integrity can be significantly compromised by telomere dysfunction, particularly in proliferating cells where cycles of instability may ensue due to creation of dicentric chromosomes (Bailey and Murnane 2006). Protein complexes that bind to the duplex and single-stranded telomere repeats are key for stabilizing the chromosome ends (de Lange 2005). In proliferating cells, this job is complicated not only because the terminal chromatin must be opened during the process of chromosome replication, but also because additional processes that metabolize DNA ends are active. For example, while nonhomologous end joining processes are preferentially used in repair of DNA double-strand breaks in G1, homologous recombination is preferentially used for this repair in S and G2 (Ferreira and Cooper 2004; Zierhut and Diffley 2008). Given these complexities, it is not surprising that our molecular understanding of how telomere proteins protect chromosomes ends is incomplete.

Budding yeast has been useful for dissecting how cells correctly metabolize their chromosome ends. In *Saccharomyces cerevisiae*, the terminal DNA comprises approximately 300 bp of $TG_{1\text{-}3}/C_{1\text{-}3}A$ sequences, ending with a short single-stranded overhang of the G-rich repeats. This 3′ overhang is ~12–14 nucleotides, although during the late S/G2 phase of the cell cycle, it becomes longer, >30 nucleotides in length (Wellinger *et al.* 1993b; Dionne and Wellinger 1996; Larrivee *et al.* 2004). Central among factors that prevent inappropriate telomere degradation in *S. cerevisiae* is Cdc13, a protein that binds to single-stranded telomere G-rich repeats (Garvik *et al.* 1995; Lin and Zakian 1996; Nugent *et al.* 1996). Reducing Cdc13 function through either the *cdc13-1* temperature sensitive (ts) allele or the *cdc13-td* conditional null (degron) allele results in telomere C-strand loss, with degradation continuing into the subtelomeric chromosomal regions (Garvik *et al.* 1995; Vodenicharov and Wellinger 2006). Correspondingly, homologous recombination at chromosome termini increases in *cdc13-1* strains (Carson and Hartwell 1985; Garvik *et al.* 1995). The loss of Cdc13 unmasks the

Supporting information is available online at http://www.genetics.org/cgi/content/full/genetics.109.108894/DC1.

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telomeres, provoking activation of the DNA damage checkpoint (Weinert and Hartwell 1993; Garvik *et al.* 1995). This protective role of Cdc13 is most likely its essential function.

A thorough, mechanistic understanding of how Cdc13 mediates chromosome end protection is hampered in part because the activities responsible for the loss of the telomere C strand are not fully known. At normal telomeres, the Mre11-Rad50-Xrs2 complex has a role regulating resection required for telomere addition, whereas the Exo1 nuclease, Rad9 and Rad24 checkpoint proteins each influence the resection process at uncapped telomeres (Lydall and Weinert 1995; Maringele and Lydall 2002; Larrivee *et al.* 2004; Zubko *et al.* 2004). The 5′-to-3′ resection of both normal and uncapped telomeres is regulated by the activity of Cdk1, the yeast cyclin-dependent kinase (Frank *et al.* 2006; Vodenicharov and Wellinger 2006). Similar to the activities that promote 5′-to-3′ degradation of DNA ends at double-strand breaks (Aylon *et al.* 2004; Ira *et al.* 2004), the activities that lead to telomere resection are active in late S and G2 cell cycle phases (Wellinger *et al.* 1993a, 1996; Marcand *et al.* 2000; Vodenicharov and Wellinger 2006). Interestingly, Cdc13 is required to prevent degradation at telomeres only in proliferating cells and not when cells are blocked in stationary phase (Vodenicharov and Wellinger 2006). Additional factors, such as the *S. cerevisiae* Rap1 protein, prevent chromosome fusions by nonhomologous recombination during the G1 phase of the cell cycle (Pardo and Marcand 2005; Marcand *et al.* 2008).

At least two additional proteins, Stn1 and Ten1, aid the capping role of Cdc13. Like *CDC13*, both *STN1* and *TEN1* are essential, and loss of their function leads to excessive single-stranded telomeric DNA (Grandin *et al.* 1997, 2001; Petreaca *et al.* 2007). *STN1* was originally identified as a high copy suppressor of *cdc13-1* temperature sensitivity (Grandin *et al.* 1997), and *TEN1* was similarly isolated as a dosage suppressor of *stn1-13* (Grandin *et al.* 2001). Combining either the *cdc13-1* allele with *stn1* mutations or the *ten1-31* allele with *stn1-13* is lethal (Grandin *et al.* 2001; Petreaca *et al.* 2007). The essential nature of these genes makes it difficult to clearly differentiate whether these genes operate in the same, or in parallel pathways to protect telomeres. A compelling argument that Cdc13, Stn1, and Ten1 likely function in a common pathway is that, in addition to these genetic interactions, Stn1 and Ten1 proteins interact with one another both *in vivo* and *in vitro* (Grandin *et al.* 2001; Gao *et al.* 2007), and each associates with Cdc13 in the yeast two-hybrid assay (Grandin *et al.* 1997, 2001; Petreaca *et al.* 2007). From these data, Cdc13, Stn1, and Ten1 are suggested to function as a single complex that mediates chromosome end protection in *S. cerevisiae*. Such a complex would share some similarities with the single-stranded DNA binding complex RPA (Gao *et al.* 2007). Whether these proteins normally operate exclusively as a heterotrimeric complex is still not entirely clear. Stn1 and Ten1 can make contributions to capping that are independent of Cdc13, as shown in experiments where overproducing the Stn1 essential domain with Ten1 replaced the essential function of Cdc13 (Petreaca *et al.* 2006). In addition, while the *Schizosaccharomyces pombe* Stn1 and Ten1 homologs are critical for telomere protection, they do not interact with Pot1, the single-stranded telomere binding protein that is also critical for telomere capping (Martin *et al.* 2007).

The role of Ten1 in maintaining both telomere integrity and length homeostasis is not understood. It has been assumed that Stn1 and Ten1 play the same role as Cdc13 in maintaining telomere integrity, namely, preventing inappropriate terminal resection. However, whether this is in fact the case is not entirely clear. For one, disrupting the DNA replication machinery can give rise to an excess of terminal single-stranded DNA, although in this case, the ssDNA accumulation is attributed to a failure to synthesize the lagging DNA strand rather than removing a block to telomere resection (Diede and Gottschling 1999; Adams Martin *et al.* 2000). Although both Cdc13 and Stn1 are thought to act as capping proteins, each can interact with Polα subunits (Qi *et al.* 2003; Grossi *et al.* 2004; Petreaca *et al.* 2006), making it important to evaluate Ten1 function more carefully. Our goal here was to compare how Cdc13 and Ten1 promote chromosome end protection, first by testing whether Ten1 acts to prevent telomere resection from activities comparable to those that degrade telomeres in *cdc13-1*, and second by determining the impact of *ten1* dysfunction upon Cdc13. The *cdc13-1* allele has been extremely useful in analyzing the *CDC13* essential function; *TEN1* analysis has been hindered by a lack of equivalent genetic reagents. Here we have created a collection of *ten1-ts* alleles useful for probing the essential role of *TEN1*. Analysis of these alleles, which show constitutive telomere elongation, reveals that Ten1 promotes telomere capping with a similar cell cycle dependency as Cdc13, protecting ends during the period in which mitotic forms of Cdk1 are active. Critically, by showing that single-stranded DNA is generated in *ten1-ts* strains under conditions where semi-conservative replication is complete, we conclude that Ten1 truly can function as a capping protein. Moreover, the *ten1-ts* strains fail to restrain degradation of chromosome ends and induce formation of Rad52 repair foci, despite the association of wild-type Cdc13 with telomeres, indicating not only that Cdc13 binds telomeres independent of Ten1 function, but also that Cdc13 telomere localization is not sufficient for end protection. Finally, although the *ten1-ts* capping-deficient phenotypes parallel *cdc13-1*, only the *ten1-ts* strains are highly sensitive to impaired *POL1* function, leaving open the possibility that *TEN1* function additionally impacts terminal replication.

TABLE 1

Plasmid list

Plasmid	Description	Reference
pCN250	*CEN URA3 native romoter TEN1*	This study
pCN284	*CEN TRP1 native promoter TEN1*	This study
pCN309	*CEN TRP1 native promoter ten1-101*	This study
pCN311	*CEN TRP1 native promoter ten1-103*	This study
pCN358	*CEN TRP1 native promoter ten1-105*	This study
pCN359	*CEN TRP1 native promoter ten1-106*	This study
pCN124	*2μ TRP1 ADH promoter GAL4 DBD TEN1*	Petreaca *et al.* (2006)
pCN445	*2μ TRP1 ADH promoter GAL4 DBD ten1-101*	This study
pCN441	*2μ TRP1 ADH promoter GAL4 DBD ten1-105*	This study
pCN447	*2μ TRP1 ADH promoter GAL4 DBD ten1-106*	This study
pCN452	*2μ TRP1 ADH promoter GAL4 DBD ten1-103*	This study
pCN181	*2μ LEU2 ADH promoter GAL4 AD STN1*	Petreaca *et al.* (2006)
pVL835	*2μ LEU2 ADH promoter GAL4 AD CDC13*$^{\Delta 585\text{-}677}$	This study

MATERIALS AND METHODS

Plasmids: Plasmids used in this study are listed in Table 1. Plasmids pCN284 and pCN250 each contain a 2-kb fragment surrounding the *TEN1* open reading frame, with 1 kb of upstream and 0.6 kb of downstream sequences. This insert was amplified from genomic DNA using primers CO13 and CO14. *BamH*I and *Hind*III restriction sites within the primers were used to subclone this fragment into YCplac22 and pRS416. Plasmids containing the mutant *ten1-ts* alleles were created by PCR mutagenesis (below). The *TEN1* two-hybrid plasmids were PCR amplified (oligos CO15 and CO16) and subcloned into pAS1.

Strains: Yeast strains were propagated following standard procedures. Strains are listed in Table 2. To knock out *TEN1*, the *KANMX2* cassette was PCR amplified from pFA6-KanMX2 (Wach *et al.* 1994) using oligos CO1 and CO2 and transformed into a diploid strain. Correct knockouts were verified by PCR. The *ten1Δ::kanMX2*/pCN250 haploids (hc558, hc1832) were obtained by dissecting the *ten1Δ::kanMX2/TEN1* [pCN250] diploid. The *ten1-ts* strains were obtained by transforming *ten1-ts* plasmids into hc1832 and shuffling out the pCN250 (*TEN1 URA3*) plasmid by plating cells on SD −Trp plates containing 1 mg/ml 5-fluoro-orotic acid (5-FOA). Double mutant strains were created through hc1832 matings, followed by tetrad dissection and plasmid shuffling.

Screen for *ten1*^{ts} alleles: Random mutations in *TEN1* were created by PCR amplifying genomic DNA with oligos CO13, CO14 under mutagenic conditions. Each 25-μl PCR reaction contained ~5 ng of genomic DNA in 2.5 mm $MgCl_2$, 0.05 mm $MnCl_2$, with 0.02 mm each of dATP and dGTP, 1.0 mm each of dCTP and dTTP, 1-μM primers, and 10 units Taq DNA polymerase (New England Biolabs). The reaction had an initial denaturation step of 2 min at 94°, followed by 35 cycles of 1 min at 94°, 2 min at 45°, 1.5 min at 72°, and finished with a final 10 min extension at 72°. pCN284 was digested with *Ppu*MI and *Bsm*I, removing a 0.6-kb fragment that contains the *TEN1* ORF. This "gapped" plasmid was purified and cotransformed with the PCR product into hc558, allowing recombination *in vivo*. Colonies on SD −Trp plates were streaked on SD −Trp 5-FOA, and tested for temperature sensitivity. pCN250 was retransformed into the ts candidates to confirm complementation by *TEN1*. *ten1-ts* plasmids were rescued and sequenced; each allele contains at least one mutation that alters the Ten1 protein sequence. Two alleles, *ten1-101* and *ten-105*, were integrated into the genome. The integrated alleles show phenotypes similar to the plasmid-borne alleles (see supporting information, Figure S1 and Figure S2).

Serial dilutions: Cultures inoculated from single colonies were incubated 4 days at 23°. For each strain, 10-fold serial dilutions from the same initial concentration of cells were done in a 96-well microtiter dish and stamped onto appropriate plates. Plates were incubated 4 days at 23°, 3 days at 30° or 36° before pictures were taken.

Southern blot: Genomic DNA was prepared as described from cultures incubated at 23° for 4 days (Lundblad and Szostak 1989). For blots analyzing telomere restriction fragments, the genomic DNA was digested with *Xho*I, fragments separated on 0.8% agarose gel, then transferred to a Hybond-XL membrane (Amersham). Blots were probed with [^{32}P]-dGT/CA and exposed on film.

Colony plating efficiency: Cells were inoculated into 5-ml SD −Trp media and incubated for 3–4 days. Cultures were then diluted and sonicated, and the concentration determined using a hemocytometer. Three hundred cells were plated on SD −Trp plates and 10^4 cells were plated on SD −Trp 5-FOA plates; for wild-type strains, 10^3 cells were plated on SD −Trp 5-FOA. The efficiency of colony formation on SD −Trp 5-FOA plates was normalized to the colony efficiency on SD −Trp plates, using the following calculation: CE_{5FOA} = number of colonies on SD −Trp 5-FOA/[10^4 × (number of colonies on SD−Trp/300)].

Telomere healing assay: The telomere healing assay was performed as described (Diede and Gottschling 1999). Cells were incubated in SD −Lys media overnight and then arrested in YP-raffinose with 15 mg/liter nocodazole for 3 hr until >80% of cells were arrested as large-budded cells. Galactose was added into the media to 3%, and the cells were harvested at 0-, 1-, 3-, and 5-hr time points. The nocodazole arrest was maintained throughout the experiment. Genomic DNA was prepared from cells at each time point and was digested overnight with *Spe*I. Southern blots were done to visualize the DNA bands, probing with [^{32}P]-*ADE2* as in Diede and Gottschling (1999). The signal on the blots was quantified with National Institutes of Health (NIH) Image J. The signals from the uncut and cut *ADE2* bands at each time point were added and normalized to the signal of the 0-hr time point. To determine the fraction of viable Ade^+Lys^- cells, aliquots from time points were spread on SD −Ade plates and grown at 23°. Colonies were replica-plated to SD −Ade and SD −Ade −Lys media.

TABLE 2

Strain list

Strain	Description	Reference
hc160	*MATa ura3-52 ade2-101 lys2-801 leu2-Δ1 his3-Δ200*	Petreaca *et al.* (2006)
hc558	*MATα ten1Δ::kanMX2* [pCN250]	This study
hc1832	*MATa ten1Δ::kanMX2* [pCN250]	This study
hc1862	*MATa ten1Δ::kanMX2* [pCN309]	This study
hc1863	*MATa ten1Δ::kanMX2* [pCN311]	This study
hc1864	*MATa ten1Δ::kanMX2* [pCN358]	This study
hc1865	*MATa ten1Δ::kanMX2* [pCN359]	This study
hc1212	*MATα ten1Δ::kanMX2* [pCN358]	This study
hc1676	*MATa cdc13-1$_{myc18x}$ ten1Δ::kanMX2* [pCN250]	This study
hc1722	*MATa rad52Δ::LYS2 ten1Δ::kanMX2* [pCN250]	This study
hc1723	*MATa est2Δ::kanMX2 ten1Δ::kanMX2* [pCN250]	This study
hc2025	*MATa ten1Δ::kanMX2* [pCN250, pCN284]	This study
hc2026	*MATa ten1Δ::kanMX2* [pCN250, pCN358]	This study
hc2027	*MATa rad52Δ::LYS2 ten1Δ::kanMX2* [pCN250, pCN284]	This study
hc2028	*MATa rad52Δ::LYS2 ten1Δ::kanMX2* [pCN250, pCN309]	This study
hc2029	*MATa cdc13-1$_{myc18x}$ ten1Δ::kanMX2* [pCN250, pCN284]	This study
hc2030	*MATa cdc13-1$_{myc18x}$ ten1Δ::kanMX2* [pCN250, pCN358]	This study
hc2031	*MATa est2Δ::kanMX2 ten1Δ::kanMX2* [pCN250, pCN284]	This study
hc2032	*MATa est2Δ::kanMX2 ten1Δ::kanMX2* [pCN250, pCN358]	This study
hc2009	*MATa rad52Δ::LYS2*	This study
hc1841	*MATa rad52Δ::LYS2 ten1Δ::kanMX2* [pCN358]	This study
hc1848	*MATa rad52Δ::LYS2 ten1Δ::kanMX2* [pCN309]	This study
hc1892	*MATa rad52Δ::LEU2 GALHO::LEU2 TG$_{1-3}$HOsite::LYS2 ten1Δ::kanMX2* [pCN250]	This study
hc1943	*MATa rad52Δ::LEU2 GALHO::LEU2 TG$_{1-3}$HOsite::LYS2 ten1Δ::kanMX2* [pCN284]	This study
hc1946	*MATa rad52Δ::LEU2 GALHO::LEU2 TG$_{1-3}$HOsite::LYS2 ten1Δ::kanMX2* [pCN358]	This study
hc1841	*MATa rad52Δ::LYS2 ten1Δ::kanMX2* [pCN250,pCN358]	This study
hc18	*MATa yku80Δ::kanMX2 CDC13$_{myc18x}$::HIS3*	Petreaca *et al.* (2006)
hc1997	*MATa cdc13-1*	This study
hc1998	*MATa cdc13-1 cdc28-as1*	This study
hc2000	*MATa ten1Δ::kanMX2 cdc28-as1* [pCN250]	This study
hc2005	*MATa ten1Δ::kanMX2 cdc28-as1* [pCN358]	This study
hc2035	*MATa CDC13$_{myc18x}$::HIS3 ten1Δ::kanMX2* [pCN309]	This study
hc1721	*Mat a RAD52-YFP ten1Δ::kanMX2 [pCN284]*	This study
hc1840	*Mat a RAD52-YFP ten1Δ::kanMX2 [pCN358]*	This study
hc 579	*Mat a exo1Δ::kanMX2*	This study
hc1970	*Mat a exo1Δ::kanMX2 ten1Δ::kanMX2 [pCN309]*	This study
hc1972	*Mat a exo1Δ::kanMX2 ten1Δ::kanMX2 [pCN358]*	This study
Hc1989	*Mat a exo1Δ::kanMX2 cdc13-1*	This study

Single-stranded TG repeat analysis: Cells were incubated at 23° overnight until OD_{600} was ~1.0. Equivalent amounts of cells were shifted to the indicated temperatures (30° or 36°) for 4 hr. For assays with synchronized cell cultures, cells were treated for 3 hours at 23° with α-factor to arrest in G1, with 200 mM hydroxyurea (HU) to arrest in S phase, and with 15 mg/liter nocodazole to arrest in G2/M phase. Only after cells had arrested were they were shifted to different temperatures. After the temperature shift, additional α-factor, HU, or nocodazole was added to the culture to keep the cells arrested. For *cdc28-as1* strains, following the arrest in G2/M induced by nocodazole treatment, 5 μM 1-NMPP1 (Calbiochem) was added to the culture and incubated at 23° for 1 hr before the temperature was shifted. Genomic DNA was prepared as described (Dionne and Wellinger 1996). One hundred micrograms of the genomic DNA was incubated with *Exo*I (New England Biolabs and USB) or mock treated with buffer prior to *Xho*I digestion. Digested DNA was separated on a 0.75% agarose gel and the nondenatured gel was probed with a [^{32}P]-dCA oligo (Dionne and Wellinger 1996). After exposure of the gel, it was denatured in a high pH buffer and reprobed with [^{32}P]-dCA oligo to measure total amount of TG_{1-3} repeats. The gels were exposed on an Amersham phosphorimager screen.

Chromatin Immunoprecipitation: Chromatin immunoprecipitations (ChIPs) were performed as previously described (Strahl-Bolsinger *et al.* 1997), with the following modifications. Two-hundred-milliliter cells were grown into OD_{600} ~1, then cultures were split into four 50-ml cultures. Two cultures were treated with 15 mg/liter nocodazole for 4 hr, with one culture at 23° and the other at 36°, while the two other 50-ml cultures were diluted to an $OD_{600} = 0.4$ and grown at 23° or 36° for 4 hr (asynchronous cultures). Cells were lysed in ChIP lysis buffer (50 mM HEPES/KOH pH 7.5, 150 mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% Na-deoxycholate), supplemented with protease inhibitors (1 mM PMSF, 1 mM benzamidine, 1mg/ml bacitracin). Ten percent of the lysate was removed for input normalization and the rest was subjected to immunoprecipitation. Immunoprecipitations were performed in 1 ml volume in siliconized tubes. Lysates were incubated with 4 μl/ml antibody (mouse monoclonal anti-MYC 9E10) overnight, followed by a 2-hr incubation with

protein A/G-Plus agarose beads (40-μl beads/sample). Beads were washed sequentially with ChIP lysis buffer, ChIP wash buffer (10 mM Tris–HCl pH 8, 0.25 M LiCl, 0.5% NP-40, 0.5% Na-deoxycholate) and 1× Tris–EDTA. DNA was isolated using a QIAGEN PCR purification kit. Tenfold serial dilutions of the isolated DNA were dot blotted onto nitrocellulose membrane and hybridized with [^{32}P]dGT/CA, the same probe used in telomere Southern blots. The blot was exposed on a Phosphoimager screen (Amersham) and quantified using the Amersham Typhoon 9410.

Western blot: Twenty-five-milliliter cell cultures were incubated at 23° overnight until the OD_{600} was ~1.0. Cells were pelleted and resuspended in 20% TCA with proteinase inhibitors, and then lysed with glass beads in a bead beater. Fifty microliters of the cell lysate was loaded onto SDS-polyacrylamide gels. For the HA-Ten1, cells were lysed using a bead beater in Buffer A (25 mM HEPES, PH 7.5, 5 mM $MgCl_2$, 150 mM KCl, 10% glycerol, and 0.5% Triton-X 100) with proteinase inhibitors; 100 μg of each cell lysate was loaded onto SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose membranes (Pierce) and probed with either an anti-myc primary antibody (9E10, Covance/Babco) or an anti-FLAG antibody (Sigma), followed by goat anti-mouse HRP conjugated secondary antibody. For loading controls, the blots were stripped and reprobed with an anti-Rad53 antibody (Santa Cruz). The Perkin Elmer Renaissance chemiluminescence reagent was used to detect the secondary antibody, and blots were exposed to Fuji film.

Yeast two hybrid: *TEN1* two-hybrid plasmids were cotransformed into pJB694a (*LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*) (James *et al.* 1996) with pCN181 (*pACT-STN1*) or with pVL835 (*pACT-CDC13*$^{\Delta 585\text{-}677}$). Colonies were inoculated in 1 ml of SD −Leu −Trp liquid media and incubated 1 day at 23°. Serial 10-fold dilutions of the cultures were then stamped onto SD −Leu −Trp, SD −Leu −Trp −Ade, and SD −Leu −Trp −His + [1mM] 3AT plates to examine *ADE2* and *HIS3* reporter gene expression. Plates were incubated 3–4 days at 23°, 30°, 32°, and 36°.

RESULTS

***ten1-ts* strains show conditional growth and constitutive interaction defects:** To develop *TEN1* reagents useful for examining its role in telomere capping, a PCR mutagenesis strategy was used to generate temperature-sensitive *ten1* alleles. Linear *TEN1* PCR products generated under mutagenic conditions were cotransformed into cells with a linear vector containing the genomic regions flanking *TEN1* at each end of the plasmid. DNA gap repair *in vivo* between the homologous sequences on the plasmid and the PCR fragments results in a circular plasmid encoding the PCR amplified *TEN1*. The four *ten1-ts* alleles that were chosen for further analysis show reproducible temperature-sensitive growth phenotypes at 36°, with maximum permissive temperatures of ~32° (Figure 1, A and B). The growth defect of each allele is recessive, being fully complemented by one wild-type copy of *TEN1* (data not shown). The *ten1-105* allele has the most severe ts growth defect; five missense mutations are distributed throughout this protein. The *ten1-106* allele has a mutation that introduces a stop codon at amino acid 121, effectively truncating 40 amino acids from the Ten1 carboxyl terminus. The only Ten1 residue altered in common with other published alleles is Q107; in *ten1-3*, Q107R was the sole lesion, and the strain showed telomere length alterations but no growth defects (Grandin *et al.* 2001). While conducting plasmid shuffle experiments, where cells retaining only the mutant *ten1-ts* plasmid are selected on plates containing 5-FOA, we noted that at 23° the plating efficiency of the *ten1*Δ/p*ten1-ts* mutant strains was >10-fold lower than that of *ten1*Δ/p*TEN1* (Table 3). This indicates that there must be some initial impediment to the viability of the *ten1-ts* strains that is overcome in the cells surviving on the 5-FOA plates.

Since Ten1 is known to associate with both Cdc13 and Stn1, a disrupted interaction among these proteins could provide a molecular explanation for the mutant phenotype. To determine whether any of the mutations in *TEN1* disrupt these potentially essential interactions, a two-hybrid assay was performed. First, all four Ten1-ts fusion proteins are expressed at similar levels, with the Ten1-106 protein migrating as expected for a truncation (Figure 1C). Wild-type Ten1 protein expression is not as robust; it is not clear if this is a factor in the apparently weaker two-hybrid interactions of wild-type Ten1 at 23° (Figure 1D). The Ten1-103 protein reproducibly shows altered mobility on SDS–PAGE; it was recently shown that mutating the adjacent residue (R47E) also impacts Ten1 gel migration (Qian *et al.* 2009). Next, Ten1 interaction was tested with Stn1 and Cdc13 across a range of temperatures. Among these four mutants, only Ten1-105 was capable of interacting with Stn1, activating each of the three reporter genes at all temperatures (Figure 1D, and data not shown). The other mutant Ten1-ts proteins failed to activate any reporter genes. None of the Ten1 mutants are competent for Cdc13 interaction (Figure 1D). Since these interactions fail even at temperatures permissive for growth, the *ten1-ts* conditional growth defects are not likely to be solely attributable to a specific interaction defect.

***ten1-ts* strains have extremely elongated telomeres:** Each *ten1-ts* strain was found to exhibit extremely elongated and heterogeneous telomeres, even at temperatures permissive for growth (Figure 2A). Thus, the telomere length defect is not conditional. To determine whether the telomeres reach a new steady-state length or whether the mechanisms that negatively regulate telomere extension are deficient, leading to continual elongation as the cells are propagated, telomere lengths were examined in strains that were serially passaged for increasing numbers of generations (Figure 2A). These data show telomeres progressively elongate with successive generations, as examined here over ~110 generations, and even continue lengthening beyond this point (data not shown). Thus, negative regulation of telomere length is severely crippled. This elongation phenotype is recessive, as shown in Figure 2B, where *ten1-105* telomeres remain wild type in length if *TEN1* is expressed from a *CEN* plasmid.

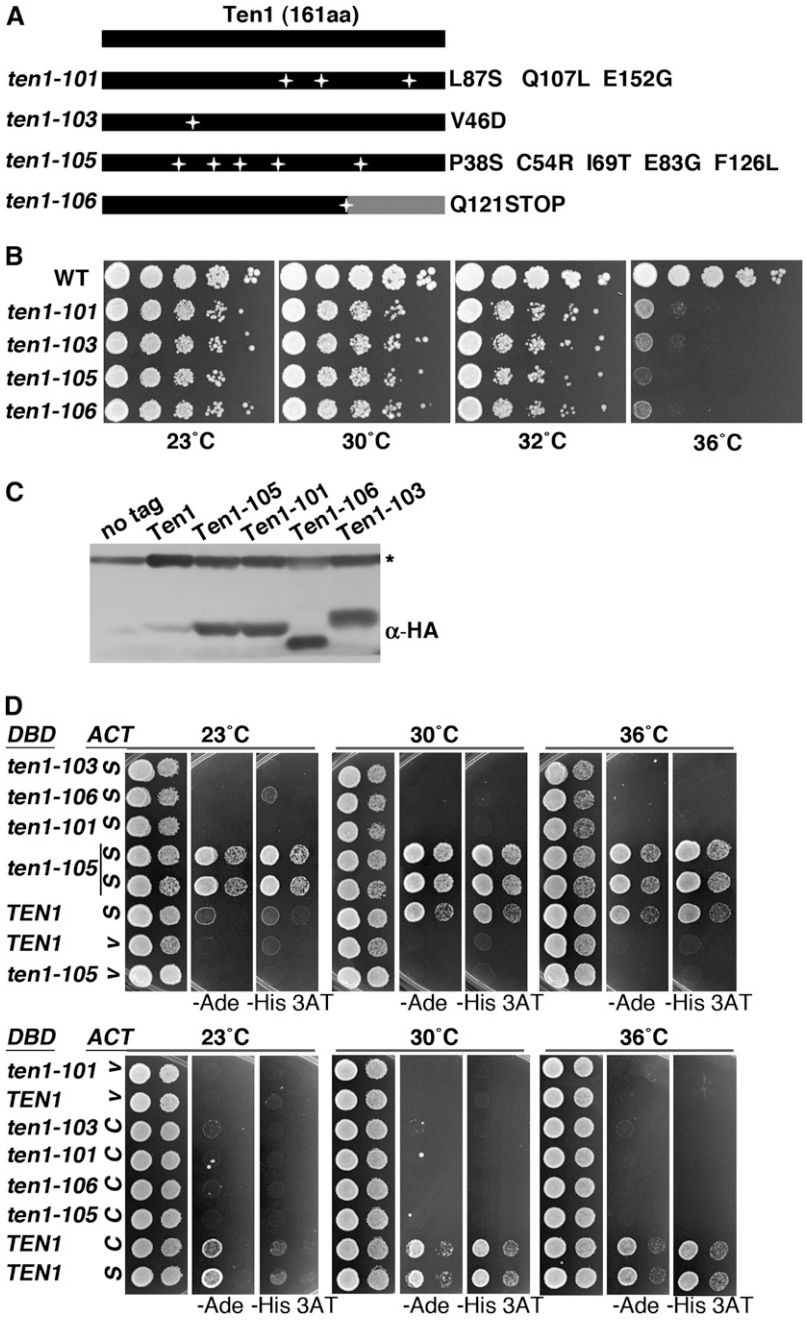

FIGURE 1.—*ten1-ts* alleles show progressive telomere elongation at permissive temperature. (A) Diagram of mutations in PCR-generated *ten1* alleles. (B) Growth phenotype of *ten1-ts* alleles. Serial 10-fold dilutions were prepared from equivalent cell concentrations and stamped onto YPD plates. The plates were incubated at the indicated temperatures for 3–4 days. WT (wild-type, hc160), *ten1*Δ*/pTEN1* (hc1832), *ten1-101* (hc1862), *ten1-103* (hc1863), *ten1-105* (hc1864), and *ten1-106* (hc1865). (C) Western blot showing expression of Ten1-ts proteins. The Ten1-ts two-hybrid plasmids were transformed into wild-type cells (hc160) and incubated at 30° overnight until the OD_{600} reached 0.8–1.0. One hundred micrograms of each cell lysate was run on a 10% SDS–PAGE gel, and each HA-$Gal4_{DBD}$-Ten1-ts fusion protein was detected by anti-HA antibody (12CA5). A cross-reacting band present in the lysates is marked with an asterisk. Strains: No tag (hc160), Ten1 (pCN124), Ten1-101 (pCN445), Ten1-103 (pCN452), Ten1-105 (pCN441), and Ten1-106 (pCN447). (D) Yeast two-hybrid analysis of interaction of Ten1-ts with Stn1 and Cdc13. Ten1 and Ten1-ts proteins were expressed as a Gal4 DNA binding domain fusion (pCN124, pCN441, pCN445, pCN447, and pCN452). *STN1* and *CDC13* were expressed as Gal4 activation domain fusion proteins (S, pCN181; C, pVL835; V, vector, pACT2.2). An in-frame deletion removes a portion of the Cdc13 DNA binding domain in pVL835 ($CDC13^{\Delta 585\text{-}677}$). Tenfold serial dilutions of cultures were stamped on SD −Leu −Trp, SD −Leu −Trp −Ade, and SD −Leu −Trp −His +1 mM 3AT plates to examine the expression of the *ADE2* and *HIS3* reporter genes.

Typically, telomerase activity is responsible for generating elongated telomeres in *S. cerevisiae*, although homologous recombination activities could also be involved. If telomerase is necessary for the *ten1-ts* telomere extension, then telomeres will remain short in telomerase-deficient strains. Conversely, if recombination is sufficient to generate and maintain the elongated *ten1-ts* telomeres, then the expectation is that loss of telomerase will not affect the length of telomeres. This was tested by creating a haploid strain, *est2*Δ *ten1*Δ*/pTEN1-URA, pten1-ts-TRP1*, which contains plasmids encoding both wild-type and mutant versions of the *TEN1* gene. When the senescing strains were plated on media containing 5-FOA to select cells bearing only the mutant *ten1-ts* plasmid, no viable colonies were obtained at 23° (Figure 2D). Similar results were obtained for all four *ten1-ts* alleles (data not shown). These *ten1-ts* strains therefore show a synthetic phenotype with the *est2*Δ null allele, preventing further growth of the senescent strain. We infer from this data that these *ten1-ts* strains are not able to elongate their telomeres independently of telomerase activity. This conclusion would be consistent with a previous analysis of *ten1* alleles, where loss of *TLC1* in a *GAL-ten1-31* strain led to telomere shortening (GRANDIN *et al.* 2001). The synthetic phenotype raises the possibility that the *ten1-ts* strains have a telomere capping deficiency at 23°, similar to *cdc13-1 est2*Δ

TABLE 3

Relative plating efficiencies of *ten1-ts* strains at 23° (percentage)

Strain genotype	*TRP1* Plasmid				
	TEN1	*ten1-101*	*ten1-103*	*ten1-105*	*ten1-106*
*RAD52 ten1*Δ	47 ± 24	2.1 ± 0.8	1.0 ± 0.6	1.2 ± 1.0	0.6 ± 0.6
*rad52*Δ *ten1*Δ	40 ± 15	0.5 ± 0.5	0.2 ± 0.3	0.2 ± 0.1	0.4 ± 0.6
*exo1*Δ *ten1*Δ	26 ± 6	5.9 ± 1.7	5.1 ± 1.9	5.9 ± 4.6	2.6 ± 1.6

Cultures were grown at 23° in −Trp liquid media for 3 days, and then the cell density was calculated by counting with a hemocytometer. Three hundred cells were plated on −Trp media and 10,000 cells plated on −Trp 5-FOA media. The number of colonies growing on the plates was determined after 7 days of growth at 23°. Multiple cultures were tested for each strain ($n = 6$). The plating efficiency was calculated for all strains, and the median fraction of cells that grow on −Trp 5-FOA relative to −Trp plates is expressed as a percentage. Thus, for *ten1-101*, only ~2% of the colonies containing the p*ten1-101-TRP1* plasmid are viable on −Trp 5-FOA plates.

strains (Nugent *et al.* 1996). In this case, the telomere shortening or lack of telomerase complex would be enhancing a *ten1-ts* protection defect present at permissive temperature.

To determine whether homologous recombination contributes to telomere length regulation, analogous plasmid shuffle experiments were conducted with *rad52*Δ *ten1*Δ strains. The *ten1-ts rad52*Δ strains are viable, with no change in their maximum permissive temperature (Figures 2D, 8A, Table 3). The telomeres in the *ten1-ts* strains become similarly elongated and heterogeneous in the absence of *RAD52* (*ten1-101 rad52*Δ shown Figure 2C), agreeing with the conclusion that telomerase creates the elongated, heterogeneous telomeres in the *ten1-ts* strains.

Cdc13 remains telomere associated in *ten1-ts* strains: Consistent with the hypothesis that *TEN1* functions in the same pathway as *CDC13*, we find that *ten1-ts* alleles have a synthetic lethal interaction with *cdc13-1* (Figure 2D). It is possible that the compromised *ten1* function directly affects Cdc13, leading to the similar capping-deficient phenotypes. Therefore, we next tested whether deficient *ten1* function alters the extent of Cdc13 association with telomere chromatin. First, we determined that the total Cdc13 protein levels are similar in both wild-type and *ten1-ts* strains at permissive and restrictive temperatures (data not shown). Next, Cdc13 localization to telomere chromatin was tested by ChIP. Since the Cdc13-mediated protection from resection is critical in G2/M arrested cells, the immunoprecipitation of telomere sequences with Cdc13-myc$_{18x}$ was tested in both asynchronous and nocodazole arrested strains. The overall telomere length is quite different in *TEN1* and *ten1-ts* strains; to correct for this, we quantified the amount of telomere repeat sequence present in the immunoprecipitate and the total lysate using dot blots hybridized with a telomere repeat probe. In addition, if Cdc13 localizes only to the ends of the telomeres, testing the association with the TG$_{1\text{-}3}$ repeats rather than an adjacent subtelomeric locus removes caveats associated with the size of the sonication fragments generated in the ChIP procedure. Analysis of multiple experiments shows that Cdc13-myc$_{18x}$ remains associated with telomeres at 36° in *ten1-101* cells at least as efficiently as in *TEN1* strains (Figure 3, A and B). No enrichment of telomere sequences in either the *Cdc13-myc$_{18x}$ TEN1* or *Cdc13-myc$_{18x}$ ten1-101* strains is observed under our experimental conditions if formaldehyde is not added (data not shown). Since the *ten1-ts* telomere repeats are greatly elongated, these data indicate that, on average, each elongated telomere is associated with more Cdc13 than is found at normal, shorter telomeres. Consistent with the increased Cdc13 telomere binding observed in *yku*Δ strains (Fisher *et al.* 2004) and in late S/G2 when telomeres transiently have longer single-stranded termini (Taggart *et al.* 2002), an increase in the single-stranded nature of the elongated *ten1-ts* telomeres would be expected to promote the overall extent of Cdc13 association in these strains. These data are consistent with the interpretation that Ten1 is not necessary for Cdc13 to bind telomeres *in vivo*, in agreement with Qian *et al.* (2009).

Ten1 helps to promote *de novo* telomere addition: Double-stranded breaks (DSB) in DNA activate a cellular checkpoint response, arresting the cell cycle and promoting activities that can lead to the repair of the broken DNA. DNA double-strand breaks are typically resected, initially being degraded from 5′ to 3′ to create single-stranded 3′ overhangs at the break (White and Haber 1990). However, it has been shown that when short tracts of telomere repeat sequences are placed adjacent to the DNA break site, the broken DNA end is protected from degradation (Diede and Gottschling 1999; Michelson *et al.* 2005; Hirano and Sugimoto 2007). The presence of this telomere "seed" sequence also greatly stimulates the addition of new telomere repeats to the DSB (Diede and Gottschling 1999). The ability of this *de novo* telomere to cap the new DNA end has been shown to be critically dependent upon Cdc13 (Diede and Gottschling 1999; Hirano and Sugimoto 2007). Since Cdc13 is proficient for telomere localization in *ten1-ts* strains and the mutant Ten1

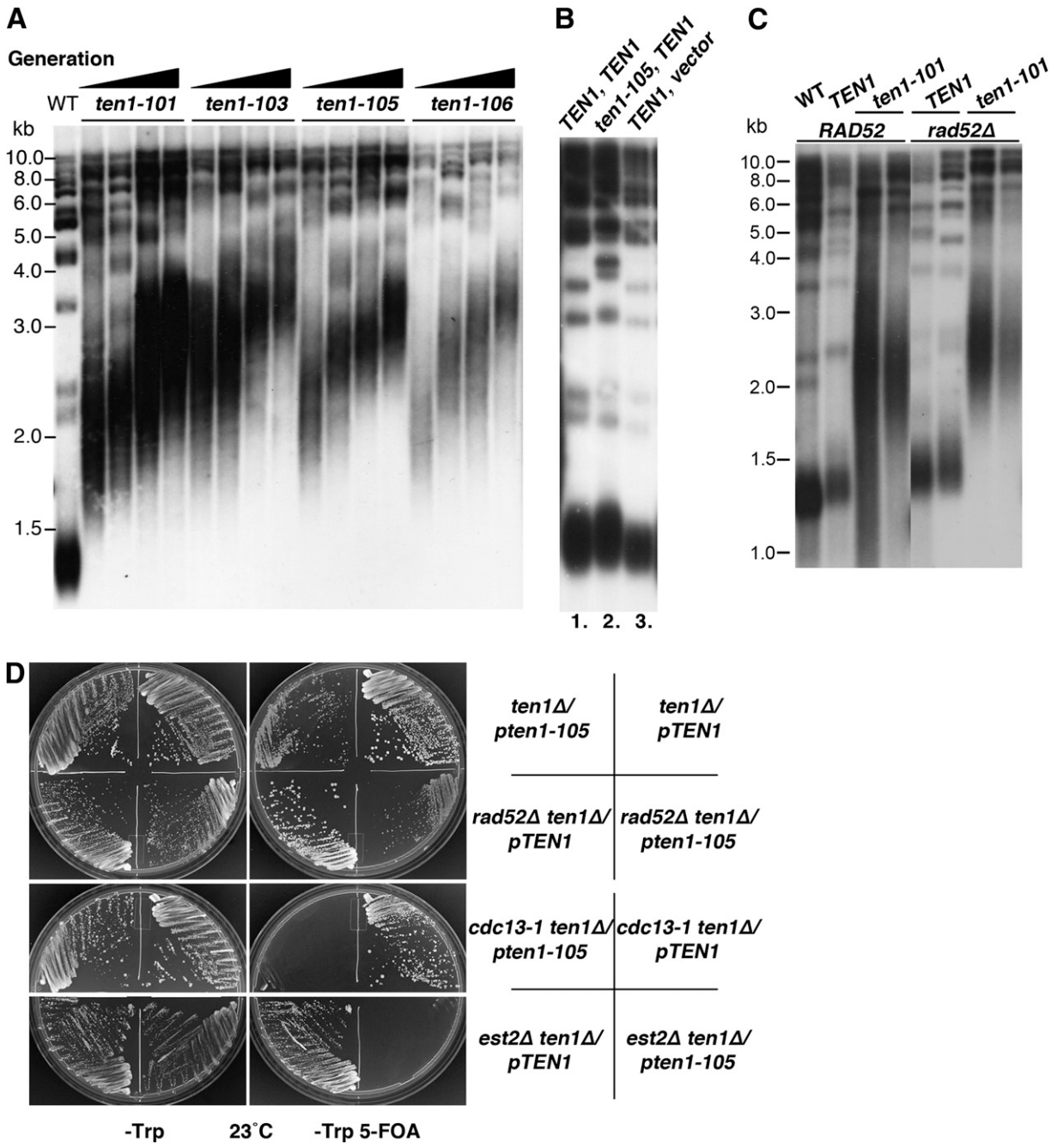

FIGURE 2.—*ten1-105* is synthetic lethal with *cdc13-1* and *est2*-Δ, and progressively elongates telomere length independent of homologous recombination. (A) Telomere length increases as strains proliferate at 23°. The initial culture for each strain (1×) was inoculated from a colony arising on SD −Trp 5-FOA plates. To obtain successive generations of the *ten1-ts* strains, colonies from the SD −Trp 5-FOA plates were propagated by successive streak-outs on SD −Trp plates. To obtain cultures of later generations, cells were inoculated from colonies arising on these SD −Trp plates. Strains were cultured in liquid SD −Trp media at 23° for 3 days. Genomic DNA was digested with *Xho*I and the Southern blot was probed with ^{32}P[dGT/CA]. WT (wild-type, hc160), *ten1-101* (hc1862), *ten1-103* (hc1863), *ten1-105* (hc1864), and *ten1-106* (hc1865). (B) Telomere length defect is recessive in *ten1*Δ*/pTEN1, pten1-105* strains. Southern blot showing *ten1*Δ strains bearing the following plasmids: lane 1, pCN250 (*TEN1*) + pCN284 (*TEN1*); lane *2*, pCN250 (*TEN1*) + pCN358 (*ten1-105*); lane 3, pCN284 (*TEN1*) + pCN416 (vector). Strains were grown at 23° for 4 days, and the Southern blot was performed as in A. (C) Southern blot comparing telomere length in *ten1-101* and *ten1-101 rad52*Δ strains. Cell cultures were incubated at 23° for 4 days. Genomic DNA was digested with *Xho*I, and the blot was probed with ^{32}P[dGT/CA]. Wild type (WT, hc160), *ten1-101* (hc1862), *rad52*Δ *TEN1* (hc1722), *rad52*Δ *ten1-101* (hc1848). (D) Plates showing growth of *ten1-105* double mutant strains that were streaked simultaneously on SD −Trp and SD −Trp 5-FOA plates. All strains initially contained the *pTEN1-URA3* plasmid (pCN250); only cells losing this plasmid will grow on the SD −Trp 5-FOA plates. Plates were incubated at 23° for 5 days. Strains: hc2026, hc2025, hc2027, hc2028, hc2030, hc2029, hc2031, and hc2032.

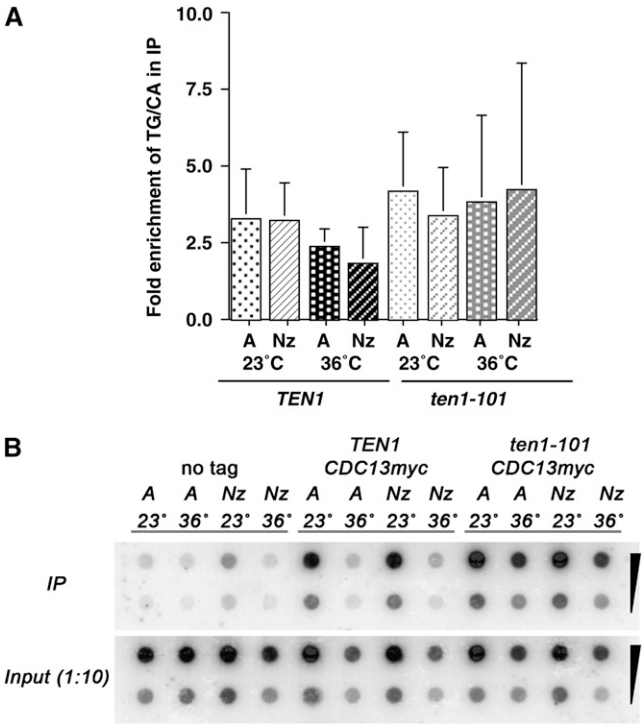

FIGURE 3.—Cdc13-myc$_{18x}$ telomere association is not diminished in *ten1-101*. (A) Chromatin immunoprecipitation of Cdc13*myc*$_{18x}$. Two hundred milliliters of wild-type untagged (hC160), *CDC13myc*$_{(18)}$ (hC1985) and *ten1-101 CDC13myc*$_{18x}$ (hC2035) strains were grown in YPD or SD −His media to an OD_{600} ~1, and split into four 50-ml cultures. Two cultures were treated with nocodazole for 4 hr at 23° and 36° (Nz), and two cultures were diluted to an $OD_{600} = 0.4$ and incubated at 23° or 36° for 4 hr (asynchronous cultures, A). Immunoprecipitations from cell lysates were carried out with an anti-*myc* antibody (12CA5). The percentage of the total telomeric DNA present in each IP was determined by dot blot analysis, hybridizing with a [^{32}P]d(GT/CA) probe, and exposing the blot on a Phosphorimager screen. To normalize the data, the percentage of telomere DNA recovered in each IP from Cdc13myc$_{18x}$ strains was divided by the percentage of telomere DNA recovered in the IP from the untagged Cdc13 strain. The averages of four independent experiments are graphed, and the error bars represent the standard deviation. (B) Dot blot of ChIP DNA samples from strains grown either asynchronously or arrested in G2 with nocodazole probed with [^{32}P]d(GT/CA), as described in A. Shown are 10-fold dilutions of the immunoprecipitate and the input DNA for each strain.

proteins are deficient for Cdc13 interaction, we next tested whether Ten1 is required to assist Cdc13 in directing telomere addition at a DNA DSB that is flanked by a telomere repeat.

Strains with an 81-bp telomere seed sequence adjacent to an HO site were used to test whether Ten1 is similarly required to protect the HO endonuclease-generated DNA double-strand breaks from resection and to promote *de novo* telomere addition (diagrammed in Figure 4A). In this system, a cassette containing the *ADE2* gene, 81 bp of TG_{1-3} repeats, and a 30-bp HO endonuclease recognition site was integrated at the *ADH4* locus near the left end of chromosome VII (DIEDE and GOTTSCHLING 1999). The HO endonuclease gene is integrated into the genome, with its expression controlled by the *GAL1* promoter, and the strain lacks the *RAD52* gene. To test telomere addition and protection of the cut end in wild-type and *ten1-105* strains, cells were arrested in G2/M using nocodazole, and the HO endonuclease was induced by switching to galactose media at the same time that the temperature was shifted to 36°. As the wild-type strain shows in Figure 4B, over time, the end that is released by the HO cut and has the adjacent telomere seed is "healed" by the addition of new telomere repeats. In contrast, telomere addition does not occur on the newly generated end in the *ten1-105* strain (Figure 4B). Moreover, after 5 hr, less of the fragment generated by the HO cut remains, even though the extent of cutting by HO is comparable to the wild-type strain. In previous studies, such diminishment of this cut end corresponded with a failure to restrain resection; strains deficient only in telomere addition retain this cut end (DIEDE and GOTTSCHLING 1999). While it is possible that in the *ten1-105* strain, extreme misregulation of telomerase creates a heterogeneous smear that is difficult to see, the simplest interpretation is that *ten1-105* cells are deficient in *de novo* telomere addition and end protection while arrested in G2/M.

To address whether the mutant strains are capable of healing the induced break if they are released from the arrest and shifted to 23°, cells from each time point were plated on −Ade. The fraction of colonies that were Ade$^+$ Lys$^-$, indicating loss of the terminal fragment, was then determined by replica plating. When the HO site is induced in cells arrested at either 23° or 36°, the proportion of the viable Ade$^+$ colonies that are "healed" at each time point is similar in wild-type and *ten1-105* strains (lines in Figure 4, C and D). This indicates that the processes required to stabilize the broken end can function efficiently in *ten1-105* strains, at least if shifted back to 23° and allowed to cycle. Nonetheless, relatively fewer *ten1-105* cells are able to form colonies after 4–6 hr, even if the HO cut is created at 23° (bars in Figure 4, C and D). The drop in the relative number of *ten1-105* cells capable of forming Ade$^+$ Lys$^-$ colonies after the shift to 36° is striking. However, a similar drop in viability is also observed when no break is created while the cells are held at 36°. If *ten1-105* cells are arrested in nocodazole and shifted to 36° for 4 hr, only ~25% of the cells remain capable of forming colonies at 23° (data not shown). Thus, in the case where the DSB is induced, the small fraction of cells capable of forming a colony may reflect lethal events other than failure to stabilize the broken chromosome end.

***Ten1-ts* strains have a conditional defect in telomere capping:** To more directly determine whether the *ten1-ts*

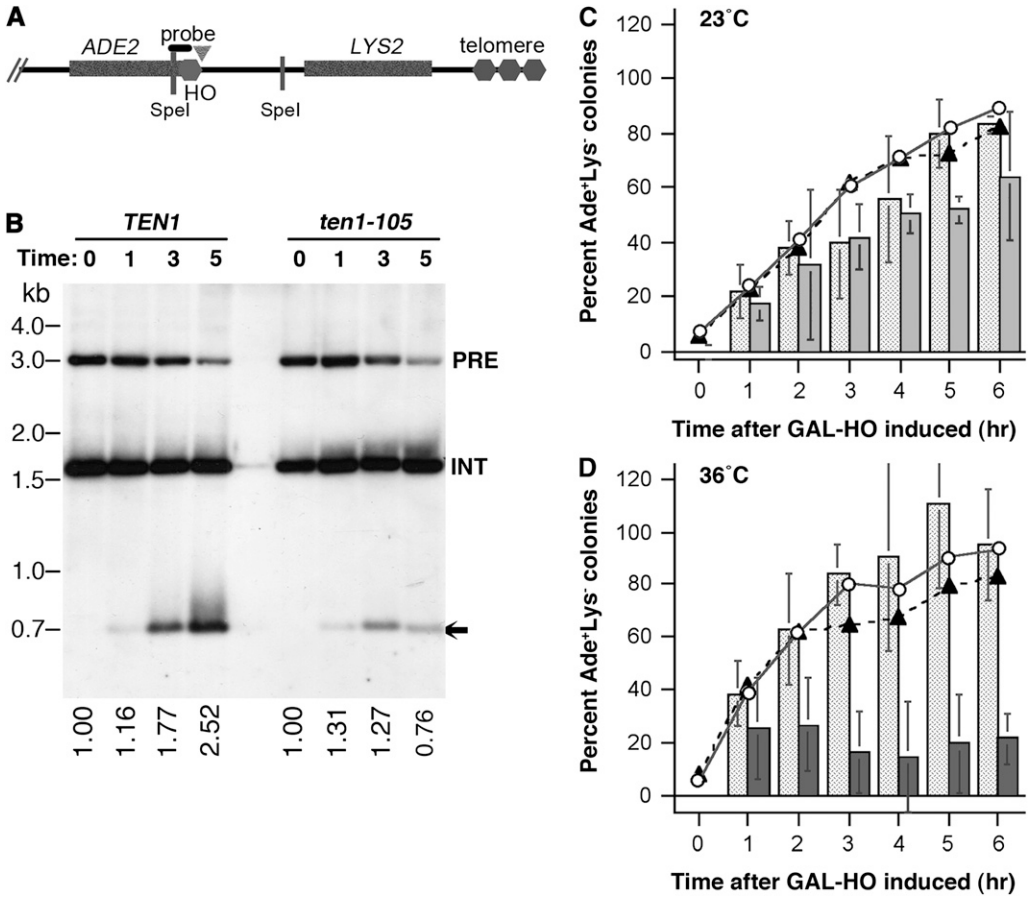

FIGURE 4.—*de novo* telomere addition requires Ten1 in G2/M arrested cells. (A) Schematic diagram of the left arm of chromosome VII containing the HO endonuclease recognition site adjacent to 81 bp of telomeric repeats (DIEDE and GOTTSCHLING 1999). The *ADE2* gene is located on the centromere proximal side of the inserted telomere repeat. The chromosome fragment from the HO site to the end of the chromosome does not contain any essential genes. A *LYS2* marker is located in this region to retain this nonessential fragment. Note: this diagram is not to scale. (B) Telomere addition at 36°. Wild-type and *ten1-105* strains containing the telomere healing cassette were grown in SD −Lys media at 23°, and then switched to YP-raffinose media containing nocodazole to arrest cells in G2/M. While maintaining the arrest, galactose was added to induce expression of the HO endonuclease, and the cultures were simultaneously shifted to 36°. Cells were collected at 0, 1, 3, and 5 hr following addition of galactose. Genomic DNA was isolated, digested with *Spe*I, and fragments were separated on a 1% agarose gel. The Southern blot was probed with a [^{32}P]-labeled fragment that hybridizes to the *ADE2* gene (DIEDE and GOTTSCHLING 1999). Both the native *ADE2* locus (INT) and the HO-adjacent *ADE2* gene are recognized by this probe. The gel was exposed on a Phosphorimager screen and the total amount of signal in the HO-adjacent *ADE2* locus determined for each lane. The numbers below each lane represent the sum of the precut and cut fragments, normalized to the amount present at $T = 0$. PRE, fragment prior to HO digestion; INT, internal *ADE2* control; arrow, fragment after HO digestion. WT (wild-type, hc1943), *ten1-105* (hc1946). (C and D) Ade$^+$Lys$^-$ colony formation as a means to assess the ability of *ten1-105* strains to heal the DSB if released from the G2/M arrest and plated at 23°. In C, cells were incubated at 23° while HO was induced; in D, cells were shifted to 36° during the induction. Colonies on SD −Ade were replica-plated to SD −Ade −Lys (and SD −Ade) after 5 days at 23°. The lines on the graphs show the proportion of Ade$^+$ colonies that are also Lys$^-$ at each time point [(Ade$^+$ Lys$^-$)/(Ade$^+$)]. *TEN1,* *ten1-105*. The bars on the graphs show the percentage of Ade$^+$ Lys$^-$ colonies relative to the number of Ade$^+$ colonies at $T = 0$; cells unable to heal the DSB are not expected to form colonies. Lighted shaded bars, *TEN1;* shaded bars, *ten1-105* solid bars, *ten1-105* .

alleles have a defect capping chromosome ends, the integrity of the telomeres in the *ten1-ts* strains was analyzed at various temperatures to test for the presence of aberrant levels of single-stranded $TG_{1\text{-}3}$ sequences. The G-rich strand was observed to be more single stranded in *ten1-ts* strains than in wild-type strains at 23° (Figure 5, A and B), consistent with the genetic data indicating a potential capping deficiency at permissive temperature. However, after incubation for 4 hr at 30° or 36°, the levels of single-stranded $TG_{1\text{-}3}$ sequences dramatically increased, indicating significantly compromised chromosome end protection at these temperatures (Figure 5, A and B). Similar conditional capping deficiencies were observed for the *ten1-101* and *ten1-106* strains (Figure 5B, and data not shown). Thus, these *ten1-ts* alleles show a severe telomere integrity defect that is temperature dependent. Taking into account the ChIP data, telomere resection is occurring in the *ten1-ts*

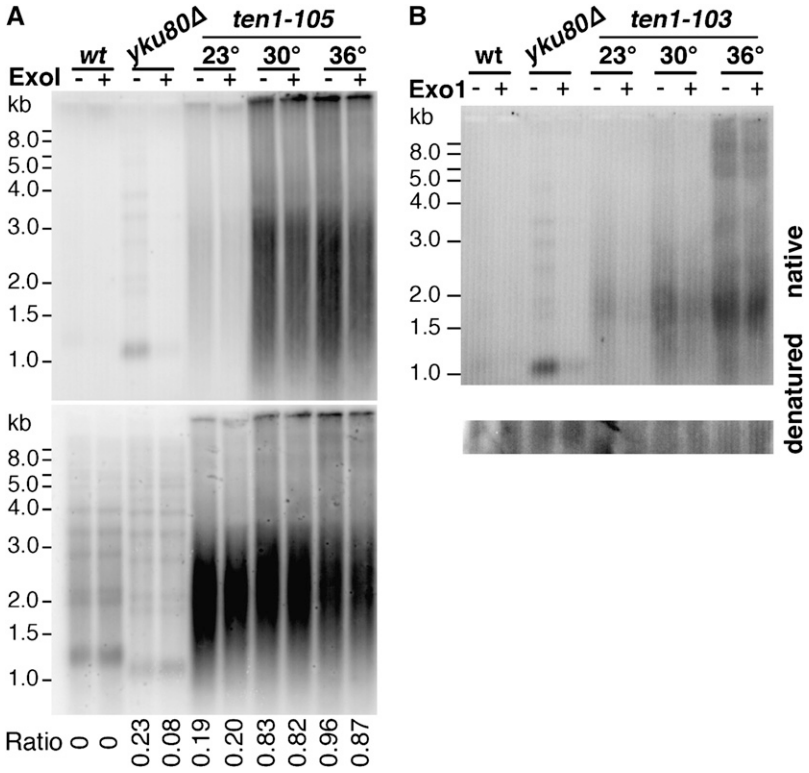

FIGURE 5.—*ten1-103* and *ten1-105* show conditional defects in telomere end protection. (A) Single-stranded telomeric DNA in *ten1-105* at 23°, 30°, and 36°. Cultures were grown at 23° overnight and then shifted to the indicated temperature for 4 hr. Genomic DNA was isolated under nondenaturing condition and split, with half treated with 40 units *Exo*I (NEB) for 2.5 hr prior to digestion with *Xho*I. The gel was probed with a [^{32}P]-labeled CA oligo that hybridizes to the G-rich telomere strand. The upper panel shows the gel under native conditions. After exposure on a Phosphorimager, the gel was denatured under alkaline conditions and hybridized with the [^{32}P]-CA oligo to measure total $TG_{1\text{-}3}$ levels. The lower panel shows the denatured gel. The number indicated below is the ratio of signal in the lane on the native gel/denatured gel, minus the ratio determined from the wild-type lanes. Strains: WT (wild-type, hc160), *yku80*Δ (hc18), and *ten1-105* (hc1864). (B) Single-stranded telomeric DNA in *ten1-103* strains at 23°, 30°, and 36°. The strains were grown, and DNA was isolated and processed as described in A. The native gel is shown in full; a representative slice from the denatured gel is shown in the lower panel. WT (wild-type, hc160), *yku80*Δ (hc18), and *ten1-103* (hc1863).

strains despite the presence of Cdc13 at telomeres. Interestingly, despite showing high levels of $ssTG_{1\text{-}3}$ at 30°, the strains remain viable, without a significant drop in plating efficiency. The increase in temperature to 36° leads to a substantial drop in cell viability and to only a small further increase in $ssTG_{1\text{-}3}$. The presence of single-stranded telomere repeat sequences in the *ten1-ts* strains is therefore not likely to be the only variable affecting cell growth at high temperature; it is possible that more internal sequences become single stranded at high temperature. At 23° and 30°, the extended telomere lengths may help mitigate an impact on cell viability.

To determine whether the single-stranded telomere repeats represent a contiguous loss of the C-rich strand from the end of the chromosome, we examined the single-stranded signal following digestion with the *Escherichia coli Exo*I enzyme. This exonuclease acts upon single-stranded 3′ DNA ends and does not degrade internal single-stranded DNA (LEHMAN and NUSSBAUM 1964). This experiment showed that the majority of the $ssTG_{1\text{-}3}$ signal in the *ten1-ts* strains is resistant to digestion, particularly at high temperatures (Figure 5). A similar resistance to *Exo*I digestion was observed for *cdc13-1* in these experiments (Figure 6). Neither overnight incubation of *ten1-105* DNA with a large excess of *Exo*I nor using an *Exo*I enzyme from a different source yielded different results (data not shown). In contrast, the $ssTG_{1\text{-}3}$ signal in the *yku80*Δ strains was sensitive to *Exo*I, as expected (GRAVEL *et al.* 1998; POLOTNIANKA *et al.* 1998) (Figures 5 and 6). The partial resistance of the single-stranded telomere regions in the *ten1-ts* strains to digestion by *Exo*I may indicate that some portion of this signal is not terminal or reflect incomplete digestion of large amounts of single-stranded DNA. Alternatively, it is possible that in the strains with longer telomeres, some DNA secondary structure forms during isolation of the DNA that obstructs access by *Exo*I. At present, we cannot resolve these possibilities. Such *Exo*I-resistant, mung bean nuclease-sensitive $TG_{1\text{-}3}$ signal has previously been observed in some *stn1* strains (PETREACA *et al.* 2007).

Ten1 is required to prevent telomere resection in G2/M arrested cells: The conditional nature of the *ten1-ts* capping defects allowed us to probe when cells require the Ten1 end protection function. As cells progress through the cell cycle, the requirements for capping chromosomes vary. If the protection Ten1 provides to telomere termini is similar to that provided by Cdc13 (and Stn1), then its capping function should be critical in the G2/M phase of the cell cycle, but not in the G1 phase (VODENICHAROV and WELLINGER 2006). Using the conditional *ten1-ts* alleles, we tested at what cell cycle stage *ten1*-deficient cells acquire aberrant single-stranded $TG_{1\text{-}3}$ DNA. Cells were first arrested at permissive temperature in G1 with α factor, in S phase with HU, or in G2/M with nocodazole treatment. After the cells had arrested, they were shifted to restrictive temperature while maintaining the cell cycle block. The analysis showed that the telomere C-strand loss in *ten1-105* cells occurs most extensively when the cells are arrested in G2/M, with little loss occurring in G1 or S phase arrested cells (Figure 6A). Similar results were

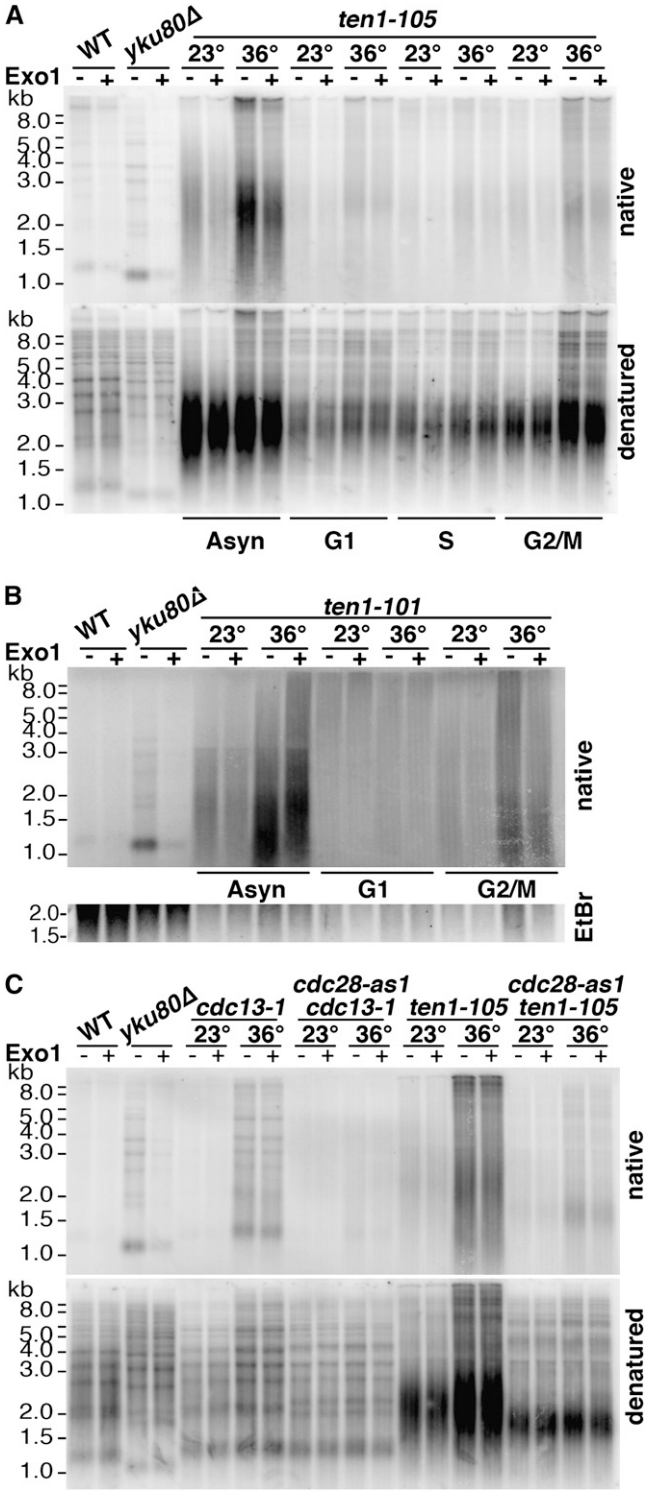

FIGURE 6.—ssTG$_{1\text{-}3}$ generation in *ten1-101* and *ten1-105* is cell cycle and Cdk1 dependent. (A) In-gel hybridization showing cell cycle dependency of single-strand generation in *ten1-105*. Asynchronous cell cultures growing at 23° were divided, with equivalent portions arrested at 23° in G1 with α-factor, in S phase with hydroxyurea (HU), or in G2/M with nocodazole. Cultures were then split and shifted to the indicated temperature for 4 hr while maintaining the arrested state. One culture was maintained in logarithmic growth prior to the temperature shift (Asyn). Genomic DNA was then isolated and split, with half treated with *Exo*I prior to *Xho*I digestion. The upper panel shows the gel probed with a [^{32}P]-CA oligo under native conditions, and the lower panel shows hybridization with the [^{32}P]-CA oligo following denaturation. WT (wild-type, hc160), *yku80*Δ (hc18), and *ten1-105* (hc1864). (B) In-gel hybridization showing cell cycle dependency of single-strand generation in *ten1-101*. Asynchronous cell cultures were divided and either allowed to continue growth at 23°, or arrested at 23° in G1 with α-factor or in G2/M with nocodozole. Cultures were then split and incubated at the indicated temperature for 4 hr while maintaining the arrested state. Genomic DNA was then isolated and split, with half treated with *Exo*I prior to *Xho*I digestion. The agarose gel was probed with a [^{32}P]-CA oligo under native conditions. Only the native gel is shown; the total DNA loaded in each lane is shown in the ethidium bromide-stained gel slice. WT (wild-type, hc160), *yku80*Δ (hc18), and *ten1-101* (hc1862). (C) In-gel hybridization showing the creation of ssTG$_{1\text{-}3}$ is dependent on functional Cdk1. Cells were arrested at 23° in G2/M with nocodazole, and then split, with half shifted to 36° for 4 hr. To inhibit the Cdc28-as1 activity, 1-NMPP1 was added to the cultures 1 hr prior to the temperature shift. The cells were arrested in nocodazole throughout the experiment. Treatment of the genomic DNA and gel probing were as in A, with the upper panel showing the native gel and the lower panel showing the denatured gel. Wild-type (WT, hc160), *yku80*Δ (hc18), *cdc13-1* (hc1997), *cdc13-1 cdc28-as1* (hc1998), *ten1-105* (hc1864), and *ten1-105 cdc28-as1* (hc2005) strains are shown.

observed for the other *ten1-ts* alleles, such as *ten1-101* (Figure 6B). It is possible that the low level of ssTG$_{1\text{-}3}$ that is detected in the G1 arrested cells arises from the small fraction of cells that fail to arrest. In the α-factor-treated *ten1-105* cultures, 85–90% of the cells arrested in G1; the small percentage of cells that did not arrest were large-budded cells (data not shown). The pattern of ssTG$_{1\text{-}3}$ generation in these *ten1-ts* strains parallels that observed in *cdc13-1* and *cdc13-td* strains (Vodenicharov and Wellinger 2006).

The cell cycle dependency of generating the single-stranded telomeres in *ten1-ts* may, like Cdc13, at least partially reflect the period of the cell cycle when the enzyme(s) that can extensively resect the telomere ends are active. In *cdc13*-deficient cells, it has been shown that telomere resection is regulated by the Cdk1 kinase (Vodenicharov and Wellinger 2006). This regulated resection activity is not active in G1, at least in response to endonuclease generated DSBs (Ira *et al.* 2004; Barlow *et al.* 2008). To determine whether the generation of single-stranded telomeres in *ten1-105* cells is controlled by Cdk1, an analog-sensitive allele of *CDC28*, *cdc28-as1*, was used. The enlarged ATP binding site in Cdc28-as1 can accommodate 1-NM-PP1, a bulky ATP analog, allowing specific inhibition of the Cdk1 activity (Bishop *et al.* 2000). As previously reported (Vodenicharov and Wellinger 2006) and shown in Figure 6C, telomeres remain double stranded in *cdc13-1* strains at 36° under conditions where Cdk1 activity is inhibited. The telomeres in the *ten1-105* strain also remain largely intact at 36° following Cdc28 inhibition (Figure 6C). Thus, Ten1 and Cdc13 protect telomeres

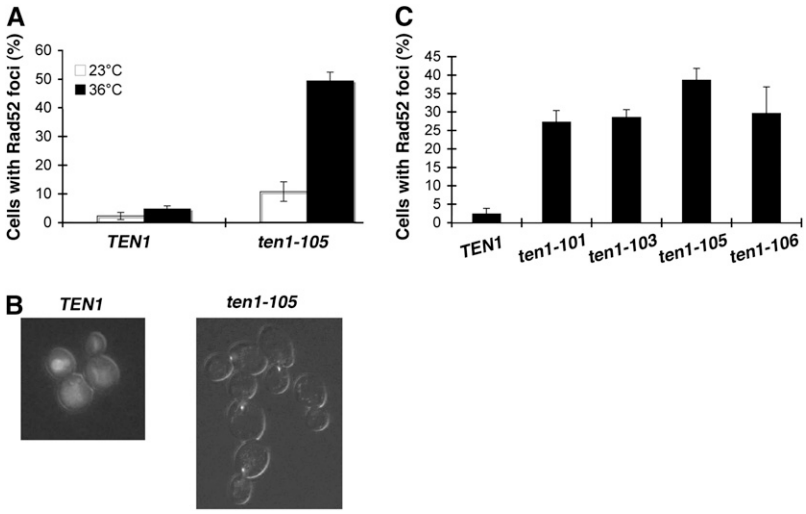

Figure 7.—Rad52-YFP foci form in large-budded *ten1-ts* cells at semi-permissive and restrictive temperatures. (A) The percentage of nocodazole-arrested cells with Rad52-YFP foci. Cells were grown at 23°, incubated in nocodazole until arrested in G2/M (3 hr), and then split, with half of the cells shifted to 36° for 4 hr while maintaining the arrest. One hundred large-budded cells per strain were scored, and the average and standard deviation from four replicates are shown. (B) Micrographs of *TEN1 RAD52-YFP* and *ten1-105 RAD52-YFP* cells were taken following overnight incubation of strains in liquid media at 30° and observed by fluorescence microscopy (Nikon Eclipse 800). (C) The percentage of large-budded cells with Rad52-YFP foci following overnight incubation in liquid media at 30°. One hundred or more cells with large buds were scored from each culture, with at least 3 cultures scored for each strain. The average number of cells with foci is graphed, with error bars showing the standard deviations. Strains: *TEN1 RAD52-YFP* (hC1720), *ten1-101 RAD52-YFP* (hc1847), *ten1-103 RAD52-YFP* (hc1857), *ten1-105 RAD52-YFP* (hc1840), and *ten1-106 RAD52-YFP* (hc1853).

from similar activities, consistent with the interpretation that these proteins provide end protection through a shared mechanism. As will be discussed below, it is possible that the low levels of $ssTG_{1\text{-}3}$ detected in the *ten1-105* strain at 36° in the inhibited Cdc28-as1 cells reflect a failure to protect from additional activities.

End protection defects correlate with Rad52 foci formation and suppression by *exo1*Δ: If Ten1 is in fact required to protect chromosome ends from resection, as indicated by the Cdk1-dependent resection of telomere DNA in *ten1-ts* mutants, it would be expected that Rad52 foci would be induced in *ten1-ts* mutants. Rad52 and other homologous recombination proteins form subnuclear repair foci in response to DNA damage such as that created by DNA double-strand breaks, single-stranded DNA, and eroded telomeres (Raderschall *et al.* 1999; Lisby *et al.* 2001; Khadaroo *et al.* 2009). To address whether *ten1-ts* inactivation can induce Rad52-YFP foci assembly after S phase, the *ten1-ts* strains were arrested in G2/M at permissive temperature, and then examined following a shift to restrictive temperature (36°). As compared to wild-type cells, the *ten1-105* strain shows a significant increase in the number of cells with Rad52-YFP foci, even in the cells incubated at 23° (Figure 7A). Consistent with the conclusion that all the *ten1-ts* alleles show a capping defect, examination of asynchronous cultures reveals that each strongly induces repair foci in large-budded cells, as shown for cells grown overnight at 30°, a permissive temperature for growth (Figure 7, B and C). The finding of substantial induction of Rad52-YFP foci in the cells even at 30° is not surprising, given the large amount of telomere single-stranded DNA that is observed at 30° (Figure 5). Nonetheless, the absence of Rad52 does not alter the viability of these *ten1-ts* strains at any temperature (Figure 8A, data not shown).

EXO1 encodes a 5′-3′ nuclease that participates in metabolism of DNA in many contexts. Of particular relevance here, Exo1 is known to contribute to the generation of single-stranded DNA in *cdc13-1*, most notably in subtelomeric X and unique regions (Maringele and Lydall 2002). Deletion of *EXO1* partially suppresses the *cdc13-1* temperature sensitivity (Maringele and Lydall 2002), consistent with a reduction in lethal single-stranded lesions created by the loss of capping. In parallel with these results, we found that *exo1*Δ is also an extragenic suppressor of the *ten1-101* and *ten1-105* temperature sensitivity (Figure 8A), now allowing cell growth at 36°. The level of single-stranded TG DNA in the *ten1-105 exo1*Δ strain is modestly reduced relative to *ten1-105* (Figure 8B). Similarly, as expected, comparatively less single-strand $TG_{1\text{-}3}$ was also observed in *cdc13-1 exo1*Δ. The simplest interpretation is that the *exo1*Δ mutation improves *ten1-ts* viability because the extent of telomere resection is reduced below a critical threshold. The observation that *exo1*Δ also improves the initial plating efficiency of *ten1-ts* strains (Table 3) suggests that *ten1-ts* cells have insufficient end protection that the cells must overcome to proliferate. This reduced ability to form an initial colony is also observed by tetrad analysis of integrated *ten1-105* alleles; ~50% of *ten1-105* cells form only microcolonies (data not shown). Further analysis of the initial events occurring in newly derived *ten1-ts* cells and the distribution and extent of single-stranded generation in the mutant strains could help resolve the mechanism of *exo1*Δ-mediated suppression.

Reduced Polα function significantly compromises *ten1-ts* growth: Our data show that the *ten1-ts* alleles phenocopy aspects of both *cdc13-1* and *stn1* mutants. Interestingly, one characteristic of *stn1* strains that

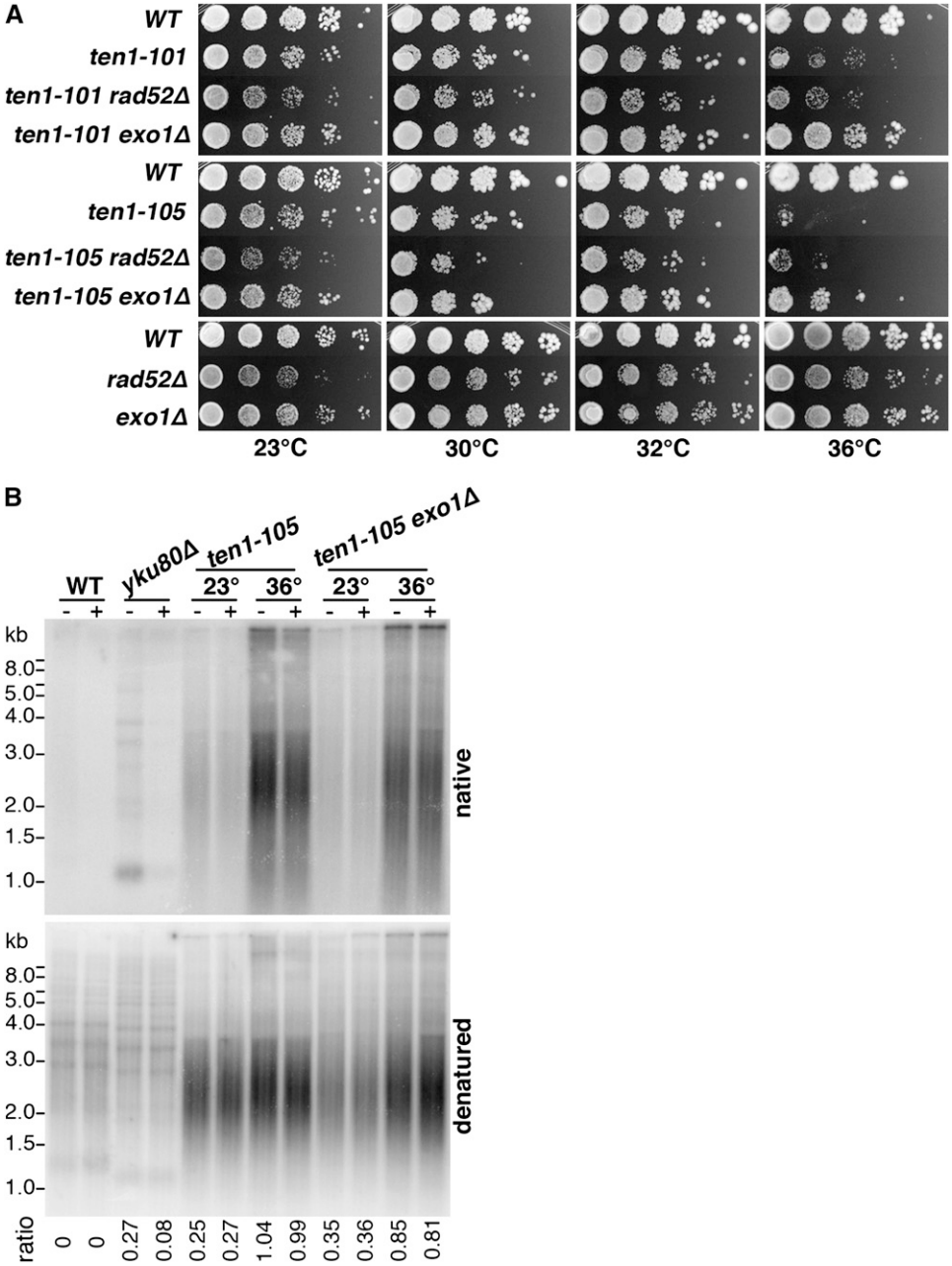

FIGURE 8.—Suppression of *ten1-ts* temperature sensitivity and TG_{1-3} resection by *exo1*Δ. (A) Temperature sensitivity of *ten1-101* and *ten1-105* are suppressed by *exo1*Δ and not affected by *rad52*Δ. Serial 10-fold dilutions were plated on YPD and incubated at indicated temperatures. Wild-type (hc160), *ten1-101* (hc1862), *ten1-101 rad52*Δ (hc1848), *ten1-101 exo1*Δ (hc1970), *ten1-105* (hc1864), *ten1-105 rad52*Δ (hc1841), *ten1-105 exo1*Δ (hc1972), *rad52*Δ (hc2009), and *exo1*Δ (hc579). (B) *exo1*Δ reduces single-stranded TG_{1-3} generation in *ten1-105* and *cdc13-1* strains. Cultures were grown at 23°, then split, with half shifted to 36°. Incubation continued at 23° and 36° for 4 hr. Genomic DNA was then isolated and half was treated with *Exo*I prior to *Xho*I digestion. The upper panel shows the gel probed with a [^{32}P]-CA oligo under native conditions, and the lower panel shows hybridization with the [^{32}P]-CA oligo after gel denaturation. The number indicated below is the ratio of signal in the lane on the native gel/denatured gel, minus the ratio determined from the wild-type lanes. Strains: wild-type (hc160), *yku80*Δ (hc18), *ten1-105* (hc1864), *ten1-105 exo1*Δ (hc1972), *cdc13-1* (hc1997), and *cdc13-1 exo1*Δ (hc1989).

appears distinct from *cdc13* strains is a strong synergistic phenotype when both *STN1* and Polα function (*POL12* in particular) are compromised (GROSSI *et al.* 2004). In particular, *stn1-13 pol12-216* strains have a synergistic phenotype, with increased levels of $ssTG_{1-3}$ generated. A similar synergism with *cdc13* was not reported. To determine whether *ten1-ts* strains are similar to, or distinct from, *cdc13* in this regard, we tested whether compromising Polα through the *cdc17-1* mutation alters the viability of *cdc13-1* or *ten1-ts* strains. As shown in Figure 9A and reported in (GARVIK *et al.* 1995), *cdc13-1 cdc17-1* strains are viable and show a slight reduction in maximum permissive temperature compared to *cdc13-1*. In contrast, the temperature sensitivity of each *ten1-ts cdc17-1* strain is greatly enhanced, with a maximum permissive temperature of ~28°, although growth is compromised even at 23°. Since *cdc17-1* does alter terminal chromatin structure (ADAMS MARTIN *et al.* 2000), it is possible that resection of telomeres is enhanced in the *ten1-ts cdc17-1* strain. To test this, the generation of $ssTG_{1-3}$ was assessed in cells arrested in G2/M prior to a shift to restrictive temperature (Figure 9B). The double mutant *ten1-105 cdc17-1* strain did not show enhanced $ssTG_{1-3}$ generation when arrested at 23° or 36°, consistent with the interpretation that resection is not enhanced *per se* in this cell cycle window. The relative level of single-stranded telomeres was similar to that in *ten1-105* (compare native to denatured signals).

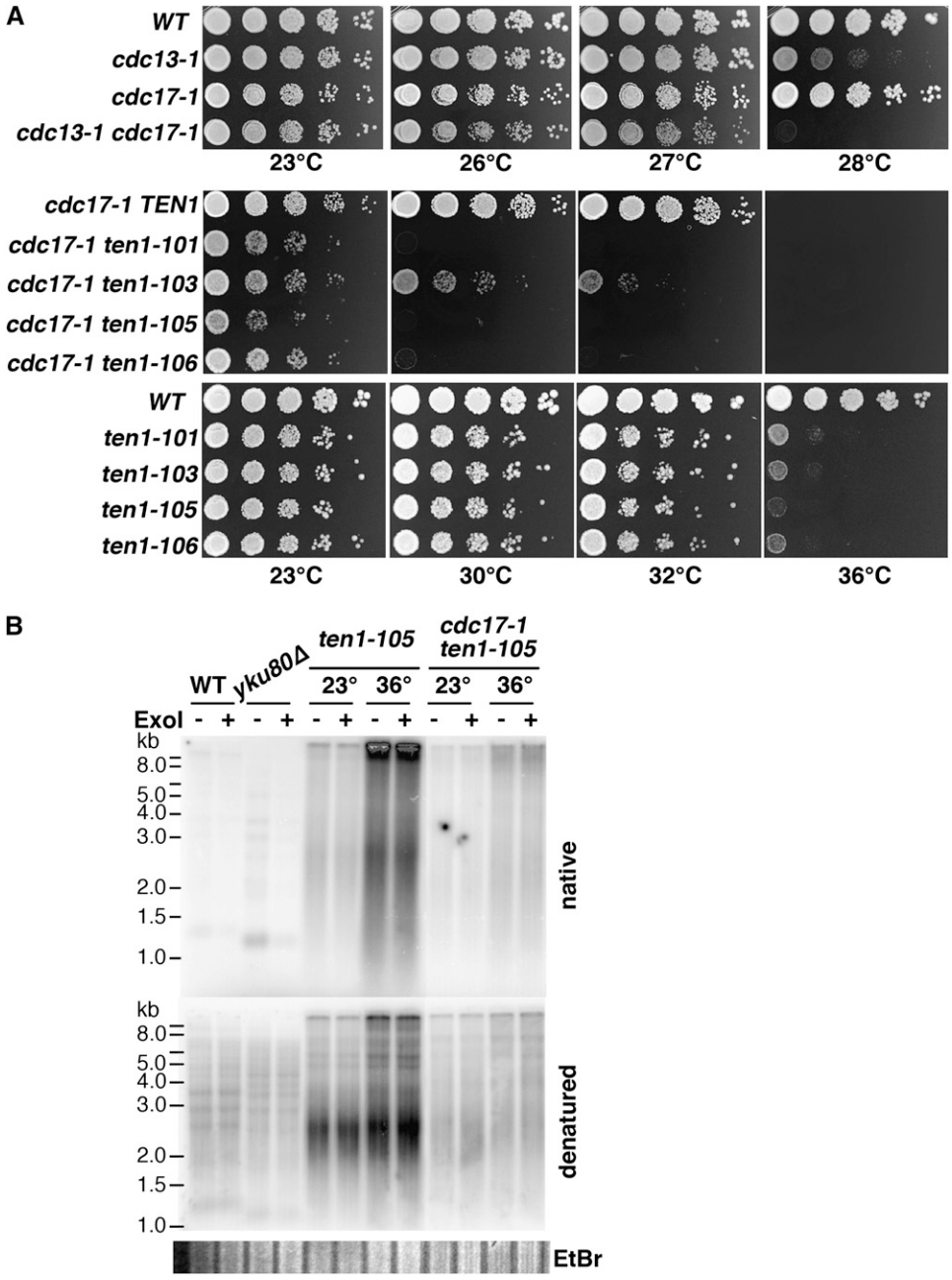

FIGURE 9.—*cdc17-1* significantly enhances the growth defect in *ten1-ts*, but not *cdc13-1* strains. (A) Growth of single and double mutants. Ten-fold serial dilutions of strains were incubated on YPD at the indicated temperatures. *cdc13-1 cdc17-1* strains were obtained by tetrad dissection. *cdc17-1 ten1-ts* strains were obtained by plasmid shuffle from parental strain hC1678, using plasmids pCN284, pCN309, pCN311, pCN358, and pCN359. The *ten1-ts* single mutant plates were incubated at the same time as the double mutants. (WT is hC160). (B) In-gel hybridization analysis of $ssTG_{1\text{-}3}$ in G2/M arrested cells. Strains were synchronized in nocodazole at 23°, and then split and incubated 3 more hr in nocodazole at 23° or 36°. DNA was isolated under native conditions; equivalent DNA concentrations were digested with *Xho*I and analyzed by in-gel hybridization. The total DNA loaded in each lane is similar, as visualized by ethidium bromide staining, shown below denatured gel.

We note that the telomere signal consistently appears more heavily amplified in the *ten1-ts* single mutant strain.

DISCUSSION

Ten1 functions as a telomere capping protein: Our data strongly support the hypothesis that Ten1, like Cdc13, protects telomeres from cell cycle-regulated activities that resect the telomere C-rich strand. Overall, the *ten1-ts* capping phenotypes parallel defects observed in *cdc13-1* strains. First, similar to *cdc13-1*, *cdc13-td*, and *stn1-td* strains (VODENICHAROV and WELLINGER 2006), extensive resection occurs when *ten1-ts* strains are blocked in G2/M, but not when blocked in G1 or in early S phase. Second, using the ATP analog-sensitive allele of *CDK1*, *cdc28-as1*, it is clear that the resection for both *cdc13-1* and *ten1-105* is dependent upon Cdk1 activity. Third, deletion of the Exo1 nuclease partially restores *ten1-ts* viability and modestly reduces the level of single-stranded telomere DNA. Finally, the loss of *ten1* function results in increased Rad53 phosphorylation (LX, data not shown), a strong induction of Rad52-YFP foci, and a *RAD9*-dependent cell cycle arrest (GRANDIN *et al.* 2001) (LX, data not shown), consistent with activation of the DNA damage checkpoint. Thus, like Cdc13, Ten1 is required to prevent resection of telomeres.

The comparable *cdc13* and *ten1* capping defects support the idea that the processes leading to creation of the single-stranded telomere repeats are similar in these strains. One difference from *cdc13-1*, however, was that despite Cdk1 inhibition in the *cdc28-as1 ten1-105* strain, a small amount of single-stranded $TG_{1\text{-}3}$ DNA was still generated at nonpermissive temperature. A trivial explanation for this would be that there was incomplete inhibition of Cdc28-as1, despite using a high concentration of 1-NM-PP1. A more speculative interpretation would be that telomere chromatin is disrupted in the *ten1-105* strain such that some Cdk1-independent resection occurs, as has been shown at a DSB in strains that are deficient for *dot1*, a methyltransferase, or for *rad9*, a checkpoint mediator that binds to modified chromatin (Lazzaro *et al.* 2008).

Does Ten1 protect telomeres only by preventing resection? While supporting a critical role for Ten1 in preventing telomere resection in G2/M, these data do not exclude the possibility that Ten1 affects telomere integrity during other cell cycle phases. We note that the extent to which telomeres become single stranded in *ten1-ts* strains is consistently higher in asynchronously dividing cells than in G2/M arrested cells. This difference could reflect the involvement of additional activities in generating the $ssTG_{1\text{-}3}$ or that the processes that are acting in G2/M have higher activity in a different cell cycle window. There is some cell cycle specificity to the operation of DNA repair pathways that can influence how a DNA double-strand break is repaired (Kanaar *et al.* 2008). By extension, the outcome of losing telomere capping is likely to be influenced by the cell cycle phase when capping is lost. Since we did not observe significant single-stranded $TG_{1\text{-}3}$ during either a G1 or early S phase arrest, we suggest that *ten1-ts* strains are likely to be highly susceptible to generating excessive single-stranded DNA during late S phase, in conjunction with the passage of replication forks through telomeres. The synthetic phenotype of *ten1-ts cdc17-1* strains is consistent with this hypothesis. Whether such single-stranded DNA would arise because Ten1 has a role connected with the conventional replication machinery in duplicating telomeres or because the *ten1-ts* defect makes telomeres more prone to resection while they are undergoing replication remains to be explored. It will be important to more fully understand how chromosome capping is maintained during DNA replication. In this regard, it is particularly interesting that the likely Stn1 mammalian homolog, OBFC1/AAF-44, has been identified as part of a complex that not only can regulate Polα activity but also associate with telomeres (Casteel *et al.* 2008; Dejardin and Kingston 2009; Wan *et al.* 2009).

What is the molecular basis of the *ten1-ts* deficiency? Since Cdc13, Stn1, and Ten1 are likely to function as a complex to promote capping (Grandin *et al.* 1997, 2001; Pennock *et al.* 2001), interactions among these proteins should be important for their function. However, three of the Ten1-ts proteins interact with neither Cdc13 nor Stn1, even at permissive temperatures. At a minimum, the Ten1–Stn1 interaction was expected to be criticial for the essential function of these proteins. The *ten1-105* allele shows a more severe phenotype than *ten1-101* or *ten1-103*, and yet Ten1-105 is competent for association with Stn1. Although the Ten1–Cdc13 and –Stn1 interactions must contribute to both telomere integrity and proper regulation of telomerase, these data indicate that the primary defect in these *ten1-ts* alleles that leads to impaired cell viability cannot simply be attributable to only a loss of interaction with either Cdc13 or Stn1.

Given the similarities between *ten1-ts* and *cdc13-1* phenotypes, it was possible that the *ten1-ts* capping defect results from deficient Cdc13 localization to telomeres. However, we found that Cdc13 remains associated with telomeres in *ten1-101* at high temperature, a condition where Ten1-101 no longer provides adequate protection. These results show that *in vivo* Cdc13 can bind to telomere repeats independent of Ten1, and demonstrate that the binding of Cdc13 to telomeres is not sufficient to provide a functional telomere cap. It was recently reported that Cdc13 association with a subtelomeric region is reduced in two *ten1* strains (Qian *et al.* 2009). The lesions in these mutants perturb the Ten1–Cdc13 contact and the strains show significant telomere elongation, but no impairment of viability (Qian *et al.* 2009). Since these sets of *ten1* alleles are all deficient for Cdc13 interaction, how Cdc13 telomere association is affected in *ten1* strains may depend more on the overall structure of the chromosome ends than specifically upon the robustness of the interaction between Cdc13 with Ten1. It would not be surprising if the distribution of Cdc13 along telomere chromatin becomes altered in *ten1-ts* strains as a consequence of the increased single-stranded nature of their telomeres.

Cdc13-bound telomeres promote *ten1-ts* viability: Ten1-ts strains rely on Cdc13 for viability even at permissive temperature. The presence of functional Cdc13 on telomeres in *ten1-ts* strains may not only lead to the extreme telomere lengthening that is observed, but also could help limit resection of subtelomeric regions. The binding of Cdc13 to the $ssTG_{1\text{-}3}$, or possibly the elongated state of the telomeres, could be the reason *ten1-ts* cells can proliferate at 30° despite significant $ssTG_{1\text{-}3}$ accumulation and strong damage foci induction. The growth defects of both *cdc13-1* and *ku* deficient strains are also modulated by telomere length (Downey *et al.* 2006; Vega *et al.* 2007). A tolerance for disrupted telomere integrity is observed in *yku70*Δ strains, which exhibit single-stranded $TG_{1\text{-}3}$ repeats at low temperatures (Gravel *et al.* 1998), and only lose viability when the temperature is raised to 37° (Feldmann and Winnacker 1993; Boulton and

Jackson 1996). In these *yku*− strains, the loss of viability correlates with both the amount of single-stranded DNA in subtelomeric regions (Maringele and Lydall 2002) and the length of the duplexed telomere tract (Gravel and Wellinger 2002). Furthermore, short tracts of telomere repeats adjacent to a DSB require Cdc13 to prevent resection, whereas long tracts of telomere repeats have a reduced dependence upon Cdc13 for protection from resection (Negrini *et al.* 2007). Both the reduced initial plating efficiency of *ten1-ts* strains (when cells initially have wild-type telomeres) and the synthetic phenotype of *ten1-ts est2*Δ are consistent with the hypothesis that increased telomere length helps compensate for *ten1* capping defects, although telomerase could also contribute to capping (Singh and Lue 2003; Vega *et al.* 2007). Thus, understanding the molecular basis for Ten1 telomere capping activity remains a significant issue for further exploration.

We are grateful to Dan Gottschling, David Morgan, and Rodney Rothstein for sharing strains. We thank lab members and Jeff Bachant for discussion and comments on this manuscript. This work was supported by a grant to C.I.N. from the National Institutes of Health (R01-CA96972).

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Communicating editor: N. M. Hollingsworth

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Supporting Information
http://www.genetics.org/cgi/content/full/genetics.109.108894/DC1

TEN1 Is Essential for *CDC13*-Mediated Telomere Capping

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DOI: 10.1534/genetics.109.108894

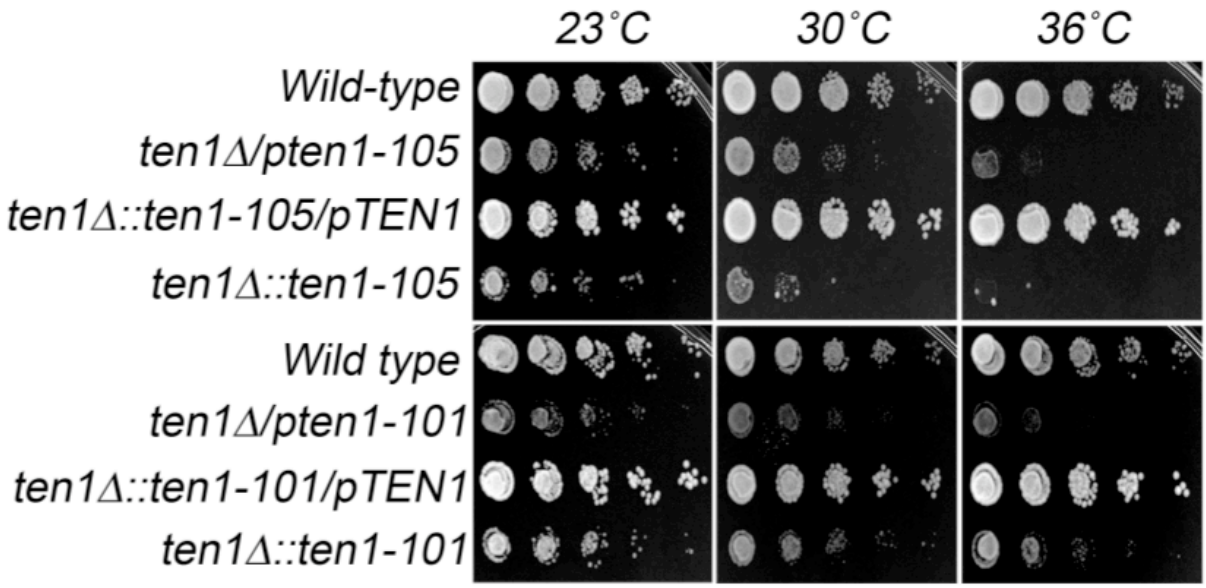

FIGURE S1.—Strains were grown overnight in YPD at 23° C. 10-fold serial dilutions were stamped onto YPD plates and incubated 3-4 days at the indicated temperatures.

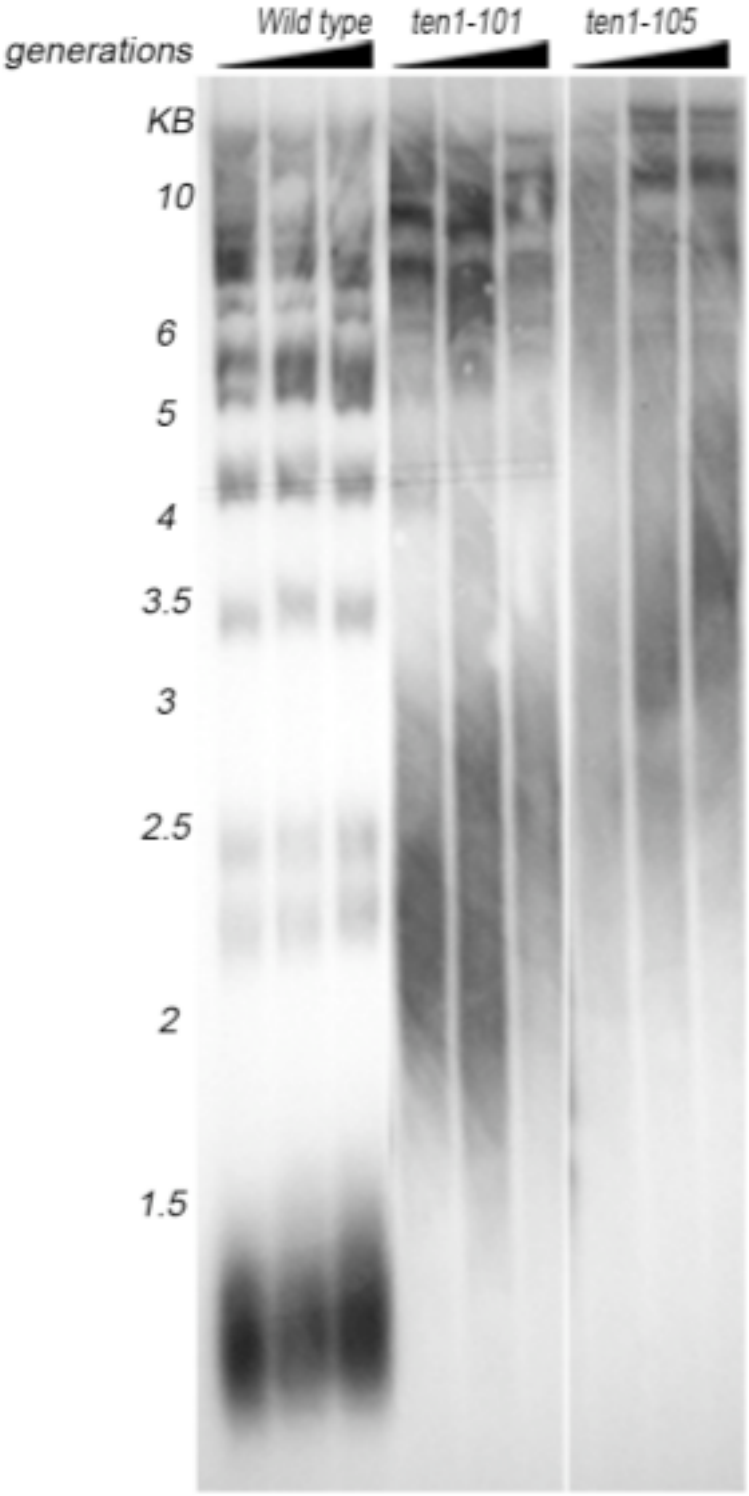

FIGURE S2.—Wild-type, *ten1Δ::ten1-101*, and *ten1Δ::ten1-105* strains were propagated by serial streak-outs on YPD plates after dissection from diploids heterozygous for the integrated *ten1-ts* alleles. 10 ml of YPD was inoculated with a colony from the 1x, 2x, and 3x plates and grown at 23°C until saturated. Genomic DNS was digested with *Xho*1, and the fragments separated on a 0.8% agarose gel. The Southern blot was probed with [32P]-TG1-3/AC1-3 probe. All lanes are from the same blot.