A naturally thermolabile activity compromises genetic analysis
of telomere function in *Saccharomyces cerevisiae*

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ABSTRACT

The core assumption driving the use of conditional loss-of-function reagents such as temperature-sensitive mutations is that the resulting phenotype(s) are solely due to depletion of the mutant protein under non-permissive conditions. However, prior published data, combined with observations presented here, challenge the generality of this assumption at least for telomere biology: for both wild type yeast and strains bearing null mutations in telomere protein complexes, there is an additional phenotypic consequence when cells are grown above 34°C. We propose that this synthetic phenotype is due to a naturally thermolabile activity that confers a telomere-specific defect, which we call the Tmp− phenotype. This prompted a re-examination of commonly used cdc13-ts and stn1-ts mutations, which indicates that these alleles are instead hypomorphic mutations that behave as apparent temperature-sensitive mutations due to the additive effects of the Tmp− phenotype. We therefore generated new cdc13-ts reagents which are non-permissive below 34°C to allow examination of cdc13-depleted phenotypes in the absence of this temperature-dependent defect. A return-to-viability experiment following prolonged incubation at 32°C, 34°C and 36°C with one of these new cdc13-ts alleles argues that the accelerated inviability previously observed at 36°C in cdc13-1 rad9-Δ mutant strains is a consequence of the Tmp− phenotype. Although this study focused on telomere biology, viable null mutations which confer inviability at 36°C have been identified for multiple cellular pathways. Thus, phenotypic analysis of other aspects of yeast biology may similarly be compromised at high temperatures by pathway-specific versions of the Tmp− phenotype.
INTRODUCTION

Telomere research in the budding yeast *Saccharomyces cerevisiae* has made substantial contributions for ~ 30 years, starting with the cloning of yeast telomeres (*SZOSTAK and BLACKBURN* 1982; *SHAMPAY et al.* 1984) and the identification of the first mutant strains with altered telomere length (*CARSON and HARTWELL* 1985; *LUSTIG and PETES* 1986). Subsequent studies have identified numerous factors which contribute to yeast telomere function. Two key complexes are telomerase (composed of the TLC1 RNA and the three Est proteins) which is responsible for elongating the G-rich strand of chromosome termini, and a heterotrimeric complex which we have called the t-RPA complex (*GAO et al.* 2007, composed of the essential genes *CDC13, STN1* and *TEN1*) which recruits telomerase to chromosome ends and also confers an essential protective function. In addition, numerous proteins share roles at telomeres and double-strand breaks (Tel1, the Mre11/Rad50/Xrs2 complex and the Ku heterodimer are three examples), and a cohort of proteins negatively regulate telomere length (Rap1, Rif1 and Rif2, as well as components of DNA replication machinery). Genome-wide efforts have expanded this list with the inclusion of several hundred additional genes (*ASKREE et al.* 2004; *GATBONTON et al.* 2006), which impact telomere function either directly or indirectly. Collectively, this very large panel of defined mutations in known genes has been the basis for numerous *in vivo* analyses of the consequences of perturbing telomere homeostasis.

In this study, we address an additional factor that impacts chromosome termini even in wild type yeast, which is the temperature at which cells are propagated. Compelling evidence for a temperature-induced impact on telomeres was first uncovered with the characterization of yeast strains bearing null mutations in either of the two subunits of the Ku heterodimer. Although viable at lower temperatures, *yku70-Δ* and *yku80-Δ* strains exhibit a *RAD9*-dependent terminal arrest phenotype at 36°, arresting after limited propagation as large budded cells (*FELDMANN and WINNACKER* 1993; *FELDMANN et al.* 1996;
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BARNES and RIO 1997). This is accompanied by a DNA damage response (BARNES and RIO 1997; TEO and JACKSON 2001), arguing that cell death is due to unrepaired DNA damage (although the molecular basis for inviability is a subject of some speculation; FELLERHOFF et al. 2000; GRAVEL and WELLINGER 2002; SMITH et al. 2008). Regardless of mechanism, the temperature-dependent change in phenotype of strains bearing null mutations in the Ku heterodimer reveals a thermolabile activity which is Ku-independent. Several other observations are also consistent with a temperature-dependent contribution, as telomeres in wild type yeast become slightly shorter when cells are propagated at 37° (GRANDIN and CHARBONNEAU 2001). In addition, est1Δ null strains have a more exacerbated growth defect when propagated at higher temperatures (LUNDBLAD and SZOSTAK 1989).

These observations suggest that the phenotype of strains with mutations in any gene affecting telomere maintenance might become more severe at higher temperature. In this study, we systematically analyzed the contribution of temperature to the growth properties of strains bearing null mutations in two key telomere complexes (telomerase and the t-RPA complex), which has revealed a pronounced impact on growth and viability in these null mutant strains at temperatures above 34°. This provides further support for a naturally occurring thermolabile activity which, when impaired at higher temperatures, gives rise to a telomere-specific phenotype, which we propose to call the Tmp− phenotype (Tmp is an abbreviation for “temperature”). This also raises the possibility that mutations in CDC13, STN1 and TEN1 which exhibit temperature-sensitive (ts) growth may not be ts for activity, but instead are partial loss-of-function mutations at all temperatures. Consistent with this, we present data indicating that the widely used cdc13-1 mutation encodes a protein which is impaired for function even at the permissive temperature of 23°, rather than a thermolabile protein. Similarly, analysis of an extensive panel of stn1Δ missense mutations, including the previously isolated stn1-13 and stn1-63 mutations, reveals a strikingly similar phenotype: defective telomere maintenance at 23° combined with impaired growth only at temperatures above 34°. We propose that this growth phenotype is due to an
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additive effect, as the result of a hypomorphic \textit{stn1}^− mutation combined with the Tmp^− phenotype, rather than a temperature-dependent impairment of \textit{STN1} function. A similar explanation potentially applies to a recently reported panel of \textit{ten1}^− mutations (Xu \textit{et al.} 2009), which we surmise may also be partial loss-of-function mutations rather than ts alleles.

Numerous prior studies with the \textit{cdc13-1} mutant strain have been performed at 36°. However, if the \textit{cdc13-1} encodes a protein which is severely impaired at all temperatures, and if a naturally thermolabile activity further impairs function above 34°, this raises questions about phenotypic analysis of \textit{cdc13-1} strains at 36°. We therefore generated a new panel of \textit{cdc13-ts} mutant strains that encode thermolabile proteins and exhibit non-permissive temperatures from 30° to 33°, thereby allowing a re-investigation of phenotypes in cells depleted for the Cdc13 protein under conditions where the Tmp^− phenotype does not contribute. Previous analysis has indicated that \textit{cdc13-1} strains rapidly become inviable following incubation at 36° through a \textit{RAD9}-dependent mechanism. However, when this experiment was repeated with one of the newly isolated \textit{cdc13-ts} strains, loss of viability in the absence of \textit{RAD9} was minimal at the fully non-permissive temperature of 32° and only became substantial at 34° to 36°. This indicates that inviability is the combined consequence of three defects (in \textit{CDC13}, \textit{RAD9} and the thermosensitive activity) and further suggests that analysis of phenotype(s) of \textit{cdc13-ts} strains at \(\geq 34°\) may be monitoring defect(s) that are not solely attributable to loss of \textit{CDC13} function.
Yeast strains and plasmids: All yeast strains, described in Table 1, were isogenic. Integrated alleles of \( \text{CDC13} \) and \( \text{STN1} \) were introduced into the genome as \( \text{URA3} \) pop-in integrants, Ura\(^{-}\) “pop-outs” were selected on 5-FOA, and the status of the \( \text{CDC13} \) or \( \text{STN1} \) locus was assessed by PCR and sequencing, to confirm that the relevant mutation was correctly integrated. A list of the plasmids, as well as the starting vectors used for each set of plasmid constructions, is shown in Table 2.

Genetic methods: Standard genetic methods (telomere length analysis, tetrad dissection, plasmid shuffle, viability assays and flow cytometry) were performed as previously described (LENDVAY et al. 1996; PASCHINI et al. 2010). For senescence assays, the relevant diploid strains were dissected, and the growth characteristics of \( tlc1-\Delta \) strains were analyzed by three successive streak-outs on rich media and scored for growth after 3 days. This assay employed very large numbers of isolates, in order to address the high degree of variability displayed by telomerase-defective strains undergoing senescence; senescence was also assessed genotype-blind for the analysis shown in Figure 8 (see GAO et al. 2010 for a more detailed discussion of this protocol).

Mutagenesis protocols: Two forward mutagenesis screens of \( \text{CDC13} \) were conducted. In the first, pVL440 (containing the intact \( \text{CDC13} \) gene) was propagated in an \( \text{E. coli} \) mutator strain as described previously (BERTUCH and LUNDBLAD 2003) to generate a mutant library of 17,000 plasmids, which was transformed into YVL3006 (a \( \text{cdc13}-\Delta/\text{p CDC13 URA3} \) shuffle strain). Transformants were subsequently screened for viability at 23\(^{\circ}\) and 36\(^{\circ}\) by replica-plating onto 5-FOA-containing media. In the second screen, a DNA fragment encompassing the DNA binding domain (DBD) of Cdc13 (amino acids 452 to 694) was subjected to PCR under error-prone conditions in the presence of 10 mM MnCl\(_2\) and one-by-one limiting concentrations of each of the four dNTPs. Pooled PCR products were co-transformed with pVL440 gapped by digestion with \( \text{BamHI} \) and \( \text{NruI} \) into the \( \text{cdc13}-\Delta/\text{p CDC13 URA3} \)
shuffle strain. The resulting 30,000 yeast transformants bearing the gap-repaired plasmids were screened for viability at 23°, 30° and 36° by replica-plating onto 5-FOA-containing media. For both screens, candidate plasmids were rescued, re-transformed to confirm the ts phenotype and subsequently sequenced. Residues for reverse mutagenesis were selected by submitting the Cdc13 DBD or the Stn1 N-terminal OB-fold domain to the Evolutionary Trace server (http://pdbjets.protein.osaka-u.ac.jp/); residues ranked in the top 10% (for Cdc13) or the top 20 residues (for Stn1) were chosen for mutagenesis.

RESULTS

Telomere shortening activity(s) are enhanced at 36° in wild type S. cerevisiae: A number of prior studies have indicated that telomere function is impaired by growth at 36° to 37°, including even telomere length in wild type yeast (GRANDIN and CHARBONNEAU 2001). We re-investigated the effect of temperature on telomere length by propagating two wild type haploid strains (which were MATa or MATα but otherwise isogenic) in liquid culture at 23°, 30°, 34° and 36° for ~100 generations. Comparison of telomere length showed that the samples grown at 30° and 34° underwent a very slight decline in telomere length, relative to the length displayed by the same cultures which had been propagated at 23° (Figure 1A). This change in telomere length homeostasis was reached within 10 generations of growth, with no further length decline upon extended propagation at the higher temperature. At 36°, telomeres underwent a further decline in length, so that the difference in length between cultures grown at 23° vs. 36° was clearly evident (Figure 1B).

There are two possible explanations for this temperature-dependent effect on telomere length: (i) elongation of telomeres by telomerase is impaired at higher temperatures or (ii) one or more processes which actively shorten telomeres is enhanced at higher temperatures. To distinguish between these two
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possibilities, the senescence phenotype of a telomerase null strain propagated at 23°, 30°, 32°, 34° and 36° was examined by successive streak-outs at each temperature, following dissection of a $TLC1/tlc1-\Delta$ diploid strain. Senescence was assessed by monitoring the growth characteristics of a large number of isolates, in order to overcome the variability of the senescence phenotype displayed by telomerase-defective strains (RIZKI and LUNDBLAD 2001; GAO et al. 2010). Figure 2A compares the growth characteristics of 42 $tlc1-\Delta$ isolates grown at 23° vs. 36° for three successive streak-outs, which demonstrates that the senescence progression was clearly exacerbated by growth at 36° even by the second set of streak-outs (which corresponds roughly to 50 generations of growth). The histogram in Figure 2B, which summarizes the relative change in the senescence score at 30°, 34° and 36° relative to 23°, shows that senescence is also accelerated at 30° and 34°, although not to the same degree as at 36°. Thus, at higher temperatures, telomeres are shorter in the presence of telomerase, and senescence is enhanced in the absence of telomerase, which is consistent with the premise that some process by which telomeres are shortened is more active at 34° to 36° (the Tmp$^-$ phenotype). We therefore propose that the growth characteristics of the $tlc1-\Delta$ strain at elevated temperatures is due to the combined result of the Est$^-$ and Tmp$^-$ phenotypes.

Microcolony growth of $cdc13-\Delta$ rad24-$\Delta$ and $stn1-\Delta$ rad24-$\Delta$ strains is reduced at 34° to 36°:

Although $cdc13-\Delta$, $stn1-\Delta$ and $ten1-\Delta$ null strains are inviable, several prior observations have suggested that the lethality of strains bearing null mutations in this complex might be partially bypassed by loss of $RAD24$ function (WEINERT et al. 1994; LYDALL and WEINERT 1995; SMALL et al. 2008). We examined this by monitoring growth of $cdc13-\Delta$ and $cdc13-\Delta$ rad24-$\Delta$ isolates following dissection of a $cdc13-\Delta/CDC13$ rad24-$\Delta$/RAD24 diploid strain. Whereas 22 $cdc13-\Delta$ strains were capable of only 1 to 2 cell divisions, all 22 $cdc13-\Delta$ rad24-$\Delta$ newly generated haploid strains underwent sufficient cell divisions to form a microcolony (a representative example is shown in Figure 3A). This behavior extended to the other subunits of the proposed t-RPA complex: 30 of 30 $stn1-\Delta$ strains and 22 of 22 $ten1-\Delta$ strains
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arrested after 1 to 2 cell divisions, whereas 29 out of 30 \textit{stn1-Δ rad24-Δ} strains and 21 out of 22 \textit{ten1-Δ rad24-Δ} strains produced microcolonies. These \textit{cdc13-Δ rad24-Δ}, \textit{stn1-Δ rad24-Δ} and \textit{ten1-Δ rad24-Δ} microcolonies were not capable of further propagation, although rare “escaper” clones could be recovered at very low frequencies (data not shown). Figure 3A further demonstrates that the ability to partially bypass \textit{cdc13-Δ} lethality was a property that extended to other members of the \textit{RAD24} epistasis group (LYDALL and WEINERT 1995; PAULOVICH et al. 1997), as \textit{cdc13-Δ rad17-Δ} and \textit{cdc13-Δ mec3-Δ} strains exhibited a comparable ability to form microcolonies.

The ability to observe a limited degree of growth in t-RPA null strains in the absence of \textit{RAD24} function provided an assay for examining whether this phenotype was also sensitive to temperature. To test this, the \textit{cdc13-Δ/CDC13 rad24-Δ/RAD24} diploid strain was dissected at 23°, 26°, 28°, 30°, 32°, 34° and 36°, and \textit{cdc13-Δ rad24-Δ} spore products were identified. Microcolony size demonstrated a reduction in size (> 2-fold) for microcolonies grown at 34° and 36° vs. lower temperatures (Figure 3B and 3C). Similar results were observed when comparing multiple \textit{stn1-Δ rad24-Δ} isolates following dissection of a \textit{stn1-Δ/STN1 rad24-Δ/RAD24} diploid strain at 23° to 36° (Figure 3D). Thus, similar to the situation with telomerase-defective yeast strains, the consequence of loss of the t-RPA complex is sensitive to elevated temperatures.

\textbf{Analysis of current temperature-sensitive alleles of \textit{CDC13} and \textit{STN1}:} Since even strains with null mutations in \textit{CDC13} and \textit{STN1} are susceptible to growth temperature, this prompted us to re-examine the characteristics of previously isolated ts mutations in these genes. In particular, we asked whether strains with previously described ts alleles displayed the properties expected for a \textit{bona fide} temperature-sensitive mutation: fully functional at permissive temperature(s) \textit{vs.} null (or greatly reduced for function) at non-permissive temperature(s).

The widely used \textit{cdc13-1} strain exhibits reduced viability at 23° and lethality at 25° - 26°. Growth is further impaired by the presence of mutations in other telomere-related genes; for example,
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cdc13-1 tlc1-Δ and cdc13-1 yku80-Δ strains are extremely sick at 23° (NUGENT et al. 1996; POLOTNIANKA et al. 1998). The severity of this synthetic phenotype suggested that Cdc13 function was impaired even at permissive temperatures. In fact, examination of steady state protein levels of the mutant Cdc13-1-(myc)18 protein compared to the wild type Cdc13-(myc)18 protein (expressed on a CEN plasmid in a wild type protease-deficient strain) did not reveal behavior consistent with a thermo-labile protein. Instead, the Cdc13-1-(myc)18 mutant protein displayed a 4-fold reduction in protein levels at 23°, relative to the wild type protein, and Cdc13-1-(myc)18 levels were not reduced further when the strain was incubated at 36° (Figure 4A). These observations argue that the cdc13-1 mutation results in a hypomorphic protein which is associated with a substantial reduction in protein levels (and presumably function) even at permissive temperatures, thereby explaining the synthetic growth characteristics. The Cdc13-1 protein may be thermosensitive as well, but the lack of change in protein levels at 36° vs. 23° suggests that at least some Cdc13 function is retained at higher temperatures. Consistent with this, in vivo association of the mutant Cdc13-1 protein with telomeres is unchanged at 37° relative to 23° (VODENICHAROV and WELLINGER 2006), arguing that the Cdc13-1 protein still retains DNA binding activity and potentially other functions at 36°. We propose that the phenotypes exhibited by a cdc13-1 strain are due to a severe hypomorphic mutation in CDC13 combined with the Tmp− phenotype, rather than due to conditional depletion of the Cdc13 protein.

For STN1, two mutations, stn1-13 and stn1-63, have been used in several previous studies (GRANDIN et al. 1997; PUGLISI et al. 2008). The stn1-13 allele (containing six missense mutations throughout the protein) is only minimally impaired for growth even at 37° (GRANDIN et al. 1997; GRANDIN et al. 2001; data not shown), and thus it was not included in our subsequent analysis. The stn1-63 strain (PUGLISI et al. 2008), which contains a single missense mutation in the essential N-terminal domain (D99E), showed a more substantial impairment for growth at 36° although the strain was not completely inviable at this temperature (Figure 4B). Somewhat surprisingly, FACS analysis
revealed that the *stn1-63* strain did not exhibit a pronounced defect in cell cycle progression at 36°C (Figure 4B), as would be expected if a subunit of the t-RPA complex had been depleted by a conditional lethal mutation. A more careful examination of the growth of the *stn1-63* strain revealed that a reduction in viability was only observed at 34°C and 36°C (Figure 4C). Steady state protein levels of the Stn1-63-(myc)₇ mutant protein were also unaffected when the culture was shifted to higher temperature (Figure 4D), in contrast to the expectations for a thermolabile protein which should be depleted (or at least diminished) at the non-permissive temperature. Furthermore, as previously observed (Puglisi et al. 2008), the *stn1-63* strain exhibited greatly elongated telomeres even at 23°C (Figure 4E), indicating that the Stn1 protein is substantially impaired even at permissive temperature. These data indicate that the *stn1-63* mutation, like *cdc13-1*, encodes a hypomorphic protein that exhibits a temperature-independent defect, rather than a thermolabile mutant protein. We hypothesize that the reduced growth at 34°C to 36°C in the *stn1-63* strain background is also an additive effect, due to a partial loss-of-function defect in *STN1* in combination with the Tmp⁻ phenotype. This hypothesis is tested further in a later section which examines an expanded panel of missense mutations in *STN1*.

Since protein levels and/or function appeared to be severely impaired at the presumed permissive temperature for the existing *cdc13* and *stn1* alleles, we conclude that these alleles are not *bona fide* conditional lethal reagents. This prompted us to screen for new ts alleles of *CDC13* and *STN1*, as described in the next sections, with the goal of identifying alleles in each gene which are fully functional under permissive conditions (such as 23°C) and completely null for function under non-permissive conditions (such as ≤ 32°C) that would be minimally influenced by the Tmp⁻ phenotype.

**Identification of new temperature-sensitive alleles of *CDC13* using both forward and reverse mutagenesis:** Two forward mutagenesis screens were performed, mutagenizing either the full length *CDC13* gene by passage through an *E. coli* mutator strain or the essential DNA binding domain (DBD) of *CDC13* (Mitton-Fry et al. 2002) by low-fidelity PCR. Both collections of mutagenized
plasmids were transformed into a cdc13-Δ shuffle strain kept alive by the presence of a covering CEN URA3 CDC13 plasmid, and yeast transformants were screened for ts growth by replica-plating onto media that selected for loss of the covering plasmid (see MATERIALS AND METHODS for details). Rescued plasmids were subsequently re-tested following re-transformation into the cdc13-Δ shuffle strain and sequenced to identify mutation(s).

Screening the mutagenized full length CDC13 gene resulted in 26 candidate cdc13-ts alleles, corresponding to seven unique mutations (Figure S1). Five alleles contained a single missense mutation: one mutation in the N-terminus of the protein (cdc13-S56F), three mutations in the DBD (cdc13-V530G, cdc13-S531F and cdc13-D546G) and one C-terminal allele (cdc13-T847M). The remaining 21 alleles had a frame shift mutation at either residue 686 (2 isolates) or residue 707 (19 isolates), resulting in truncation of the protein just past the boundary of the DBD (with either 10 or 65 amino acids added to the end of the protein as a result of the frame shift mutation). Recovery of these two frame shift mutations, as well as cdc13-T847M, was somewhat unexpected because a previously well-characterized allele of CDC13 (cdc13-5), which contained a stop codon introduced at amino acid 694, does not exhibit thermolabile growth (CHANDRA et al. 2001 and Figure S2).

The second screen, which targeted the DBD of CDC13, yielded 18 alleles with ts phenotypes. Sequence analysis revealed that all but two of these alleles had multiple missense mutations, a common problem with error-prone PCR protocols. However, several clusters of amino acids (aa 525 to 544, 611 to 618 and 683 to 684) appeared to be over-represented (Figure S2), suggesting that this information might be useful in identifying the causative mutation for at least a subset of these 18 isolates. Therefore, a panel of single missense mutations in residues in these clusters was constructed and tested for ts growth. This analysis identified 6 alleles – cdc13-L529Q, cdc13-V543F, cdc13-S611L, cdc13-G614V, cdc13-F683L and cdc13-F684S – which conferred a ts phenotype, with impaired growth at temperatures ranging from 30° to 36° (Figure S1).
In parallel with these two forward mutagenesis screens, we also employed reverse mutagenesis, using a computational method called Evolutionary Trace (ET) to identify residues in the DBD domain as targets for reverse mutagenesis. ET combines structural information with amino acid diversity to determine the evolutionary pressure at a given residue, which can identify functionally significant residues (LICHTARGE et al. 1996; LICHTARGE and SOWA 2002). A total of 14 amino acids in Cdc13 with an ET score of $\leq 10\%$ were selected for mutagenesis (excluded from this set were residues that contact DNA, which are under analysis in a separate study in this laboratory). Each residue was mutated to alanine, and the resulting collection of plasmids were introduced into a cdc13-Δ shuffle strain and assayed as described above. Four of these 14 strains displayed growth defects: one allele, cdc13-D546A, conferred lethality (data not shown), whereas strains expressing alanine mutations in 3 residues (F547, N609 and F684) exhibited ts growth (Figure S1).

Collectively, these three screens yielded missense mutations in 13 amino acids of Cdc13 which conferred conditional lethal growth. To determine which of these cdc13-ts alleles were fully functional at permissive temperatures but null with regard to both phenotype and protein levels at higher temperatures, we initially examined cell cycle progression at 23° and 36°. Two mutants (cdc13-V543F and cdc13-T847M) did not exhibit a complete cell cycle arrest at 36°, and one mutant (cdc13-S56F) had a slight cell cycle delay even at 23° (data not shown); these three mutants were discarded from further analysis. As a next step, seven mutants which were fully viable at temperatures above 28° were examined for Cdc13 protein levels (mutant strains with defects in viability at $\leq 28°$ were not analyzed, on the assumption that this would correlate with reduced function and/or protein levels even at permissive temperatures). Each of these seven mutations were introduced into a plasmid construct expressing Cdc13-(myc)$_{18}$, and extracts prepared from strains expressing these mutant Cdc13-(myc)$_{18}$ proteins grown at 23° and 36° were examined for protein levels by western analysis. In contrast to the Cdc13-1 protein (Figure 4A), all seven mutant proteins behaved like thermolabile proteins, with protein
levels substantially reduced at 36°, relative to protein levels at 23° (Figure 5A). However, two mutant proteins displayed > 3-fold reduction in protein levels even at 23° (\textit{cdc13-F547A} and \textit{cdc13-L529A}) and were excluded from the next stage of analysis.

Each of the remaining five mutations were integrated into the genome in place of the wild type \textit{CDC13} gene for subsequent analysis, to exclude possible artifacts due to variations in plasmid copy number and/or altered gene expression by plasmid-borne alleles. The resulting \textit{cdc13-ts} strains were screened for effects on viability and cell cycle progression at a range of temperatures between 23° and 34°. Cell viability assays demonstrated that these new \textit{cdc13-ts} alleles were fully viable at 28° and inviable at temperatures ranging from 30° to 33° (Figure 5B). It is also worth noting that each of the resulting integrated strains exhibited a ts phenotype which was slightly more severe than the comparable mutation when examined on a \textit{CEN} plasmid in a \textit{cdc13-\Delta} null strain (compare Figure S1 with Figure 5B). This parallels previous comparisons from our laboratory of the viability of the \textit{cdc13-1} allele as an integrated vs. plasmid-borne allele (data not shown), as well as comparisons of integrated vs. plasmid-borne \textit{stn1}\textsuperscript{−} and \textit{ten1}\textsuperscript{−} alleles (\textit{PASCHINI} \textit{et al.} 2010).

A return-to-viability experiment demonstrates that phenotypes of \textit{cdc13-ts} strains at 34° to 36° are not solely due to loss of \textit{CDC13} function: These \textit{cdc13-ts} mutant strains provide a set of reagents for analysis of \textit{CDC13} under conditions where Cdc13 is functional at 23° and fully impaired at temperatures below 34° to 36°. As a first step in assessing this, we repeated a return-to-viability experiment that has previously been used to analyze Cdc13-related defects in the presence or absence of \textit{RAD9} function in a \textit{cdc13-1} strain (\textit{WEINERT} and \textit{HARTWELL} 1993; \textit{LYDALL} and \textit{WEINERT} 1995; \textit{ADDINALL} \textit{et al.} 2011). In this protocol, \textit{cdc13-1} cells grown at 23° are shifted to non-permissive temperature for varying time periods and subsequently assessed for viability at permissive temperature (23°). Prior versions of this experiment have used 36° or 38° as the non-permissive temperature, whereas in the experiment presented here, the \textit{cdc13-S611L} strain was incubated at 32°, 34° or 36°. As
expected based on the observations in Figure 5B, cdc13-S611L cells failed to undergo cell division when incubated for 8 hours at 32°, which was accompanied by a cell cycle arrest. The response at 32° was indistinguishable from that at 34° (Figure 6A and 6B), confirming that 32° was fully non-permissive for this cdc13-ts strain.

Consistent with previous analysis of the cdc13-1 strain (WEINERT and HARTWELL 1993), arrest of the cdc13-S611L strain resulted in no more than a 1.5-fold loss of viability, even after eight hours at 32°, 34° or 36° (Figure 6A and data not shown). As was also expected from prior observations, loss of RAD9 function prevented cell cycle arrest when the cdc13-S611L rad9-Δ strain was incubated at 32° (Figure 6B). Incubation of cdc13-S611L rad9-Δ cells at the non-permissive temperature (32°) was accompanied by modest reduction in viability (5-fold by 8 hours). However, when cells were incubated at 34°, there was a striking effect in the absence of RAD9 function, as viability was reduced by 65-fold at the 8 hour time point (Figure 6A). Increasing the incubation temperature to 36° reduced viability even further (data not shown). Thus, the RAD9-dependent effect at 34° to 36°, which was well above the non-permissive temperature for the cdc13-S611L strain, indicated that an additional defect which is independent of CDC13 function, but RAD9-dependent, contributed to inviability at 34° to 36°.

**Reverse mutagenesis of the essential N-terminal domain of STN1:** In an attempt to recover conditional lethal alleles of STN1, we employed two reverse mutagenesis strategies which targeted the essential N-terminal domain of the protein. Because the Evolutionary Trace (ET) protocol was so effective with CDC13 (3 out of 14 mutations yielded a thermolabile protein, including two of the mutations shown in Figure 5), ET was similarly applied to the predicted OB-fold domain of Stn1. The top 20 residues were mutated to alanine, and a subset were also mutated to serine. These 30 stn1 missense mutations were transformed into a stn1-Δ/p CEN URA3 STN1 strain, and yeast transformants were screened for ts growth following loss of the covering plasmid (Figure S3 and data not shown). Roughly half of these mutant strains had no noticeable growth defect and were eliminated from further
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analysis. The remaining strains exhibited a range of growth phenotypes, which fell roughly into three
categories. One subset (stn1-N67A, stn1-E167A, stn1-W171S, stn1-L181S stn1-L70A, stn1-I73S and
stn1-G77A) grew approximately as well as wild type at lower temperatures but exhibited growth defects
at 34° to 36° (Figure S3); however, examination of telomere length revealed that substantial telomere
elongation had occurred even when these strains were grown at permissive temperature (Figure S4 and
data not shown). Strains in the second category (stn1-L41S, stn1-F64A, stn1-F64S and stn1-L70S) were
somewhat impaired for growth at temperatures ranging from 23° to 34°, with the growth phenotype
more severe at 36°; all four of these strains exhibited even more extensive telomere elongation at 23°
(Figures S3 and S4). The last category (stn1-G77S, stn1-D98A and stn1-D99A) exhibited the most
severe growth defect, as all three strains were barely viable at temperatures up to 32° to 34° and inviable
at 36° (Figure S3). In every case, the growth defect associated with a given mutation became more
pronounced at 36°, a pattern that was very similar to that described above in Figure 4 for stn1-63.
Furthermore, cell cycle progression as assessed by FACS demonstrated that the reduced growth at high
temperatures did not exacerbate the relatively modest cell cycle defect displayed by most of these
mutants (Figure S3 and data not shown). Thus, this first attempt at recovering one or more ts alleles of
STN1 appeared to be unsuccessful.

However, inspection of the position of this collection of mutations on the predicted structure of
the Stn1 protein suggested a possible structural correlation: mutations with the most severe growth
defects were located in residues that comprised, or were in close proximity to, the β-barrel of the OB-
fold (G77, L70, D98, D99). This suggested that mutagenesis that targeted this particular region of the
Stn1 protein might be more successful. Specifically, we directed our attention to a panel of 11
hydrophobic residues (I73, L75, I79, I93, L97, L106, L140, V142, L153, V155 and L158) with side
chains located in the interior of the β-barrel of Stn1, on the assumption that (partial) destabilization of
the OB-fold might have a higher probability of generating thermo-sensitive proteins. Each of these 11
residues were mutated to alanine, serine and tyrosine (based on the results described above for Cdc13, which indicated that restricting mutagenesis to alanine missense mutations might be insufficient). The resulting panel of 33 plasmids bearing stn1 missense mutations in the β-barrel were introduced into a stn1-Δ shuffle strain and examined at a range of temperatures from 23° to 36°. Not unexpectedly, a large number (27%) of the resulting strains were inviable or nearly inviable. Many of the viable strains exhibited a range of growth defects and once again, the severity of the defect was enhanced in each case when the strains were propagated at 34° to 36° (summarized in Figure S5). Furthermore, the majority of the viable strains exhibited elongated telomeres even when the strains were propagated at permissive temperatures (Figure S4). Only two residues, L106 and L140, appeared to be immune to mutagenesis, as the strains expressing mutations in either amino acid exhibited wild type growth at all temperatures with no telomere length defect, despite the fact that these two bulky hydrophobic residues were predicted to be on the interior of the barrel of the OB-fold (data not shown).

**Analysis of integrated stn1 alleles:** Very few, if any, of the panel of stn1 mutations described above behaved as expected for a thermolabile protein. However, in a previous study, we observed differences in viability when comparing stn1 alleles present on a plasmid in a stn1-Δ strain vs. integrated into the genome (PASCHINI et al. 2010). Since this current set of stn1 mutations were also expressed on a CEN plasmid in a stn1-Δ strain, we considered the possibility that fluctuations in plasmid copy number might mask a ts phenotype. To test this, diploid strains which were heterozygous for STN1 were constructed by integrating candidate stn1-ts alleles into the genome (see MATERIALS AND METHODS for details), and haploid stn1 strains were generated by dissection. Five alleles (stn1-I73A, stn1-I73S, stn1-I79S, stn1-G137A and stn1-G137S) were chosen for this analysis, based on the magnitude of the difference comparing growth at 23° vs. 36° when assessing the plasmid-based phenotype in a stn1-Δ strain (Figures S3 and S5).
Dissection of the *stn1-I73S/STN1* diploid revealed that the haploid *stn1-I73S* strain was inviable, as germinated *stn1-I73S* spores were capable of undergoing only 1 to 2 divisions even at 23° (Figure 6A). This indicates that *stn1-I73S* is a null mutation, since *stn1-I73S* and *stn1-Δ* strains resulted in the same phenotype following dissection. Furthermore, germinated *stn1-I73S rad24-Δ* spores were capable of forming microcolonies (Figure 6A), similar to our observations for *stn1-Δ rad24-Δ*. Therefore, the apparent ts plasmid-based phenotype (viability at 23° to 32° and inviable at ≥ 34°) was not due to a thermolabile protein; consistent with this, western analysis did not reveal any change in steady state levels of the Stn1-I73S protein when examined by western analysis from extracts grown at 23° versus 36° (data not shown). We postulate that viability of the *stn1-Δ* strain expressing the plasmid-borne *stn1-I73S* mutation was due to increased plasmid copy number in response to selective pressure for viability.

In contrast, dissection of the other heterozygous strains gave rise to viable *stn1−* haploid strains. Among this set of four strains, only the *stn1-I73A* strain exhibited a possible thermosensitive phenotype, as the mutant strain exhibited a noticeable growth defect at 30° compared to 23°, although growth was once again more severely affected at 34° to 36° (Figure 6B). This slow gradient of impairment suggested that the *stn1-I73A* mutation might encode a partially defective protein even at permissive temperatures. Consistent with this, telomere length was substantially affected in the *stn1-I73A* strain even at 23°, although telomeres were further elongated when the strain was propagated at higher temperatures (Figure 6C). Thus, we conclude that *stn1-I73A* encodes a protein which is most likely both hypomorphoric and thermolabile, with a partial loss of function at permissive temperature which is further exacerbated by growth at higher temperatures.

**Re-examination of a *cdc13-1* suppressor reveals a complex genetic interaction between null mutations, cold-sensitivity and the *Tmp−* phenotype:** Several genome-wide screens for genes that enhance or suppress the ts growth of a *cdc13-1* strain have yielded several hundred genes, implicating a wide number of molecular pathways in telomere capping (Downey *et al.* 2006; Addinall *et al.* 2008).
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The analysis above indicates that temperature alone can impact telomere-related phenotypes, which adds an additional layer of complexity when interpreting the results from *cdc13-1*-based screens. To investigate this more closely, we re-examined one candidate (*CGI121*) recovered from a *cdc13-1* suppressor screen. Since loss of Cgi121 function is capable of rescuing the temperature-dependent growth defects displayed by the hypomorphic *cdc13-1* strain and a *yku80*Δ null strain ([Downey et al.](#) 2006), we asked whether a *cgi121*Δ mutation would have a similar impact on the senescence phenotype of a *tlc1*Δ strain or the microcolony growth of a *cdc13*Δ *rad24*Δ strain.

Dissection of *cgi121*Δ/*CGI121* diploids revealed an unexpected surprise, however, which was that the *cgi1*Δ strain was itself cold-sensitive for growth. A comparison of colony sizes for *CGI121* vs. *cgi121*Δ strains following sporulation at 23°, 30° and 36° demonstrated that the *cgi121*Δ strain exhibited a substantial growth defect at 23° which was largely alleviated by growth at 36° (Figure 8A). A re-examination of prior published data by Durocher and colleagues indicates that a cold-sensitive growth defect had been previously observed for *cgi121*Δ as well as for a strain with a null mutation in *BUD32* (another member of the same complex ([Downey et al.](#) 2006), supporting the results reported here. Thus, the growth phenotype of a *cgi121*Δ strain is the consequence of two activities: loss of Cgi121 function combined with an activity which is naturally impaired for function at 23° but not 36°.

To assess for effects on senescence, *tlc1*Δ and *tlc1*Δ *cgi121*Δ strains were propagated for ~75 generations following dissection at either 32° or 36° (at lower temperatures, the significant growth defect due to *cgi121*Δ overwhelmed the senescence phenotype). As shown in Figure 8B, loss of Cgi121 function significantly attenuated senescence at both temperatures. Thus, a null mutation in *CGI121* acts as a partial bypass suppressor of a null mutation in telomerase, at least at high temperatures. In contrast, the genetic interaction between null mutations in *CGI121* and *CDC13* was more complex. At 23°, the *cgi121*Δ mutation was capable of bypassing the near-lethality of a *cdc13*Δ *rad24*Δ strain: *CDC13* was no longer an essential gene in a *cgi121*Δ *rad24*Δ background, such that
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*CDC13 cgi121-Δ rad24-Δ* and *cdc13-Δ cgi121-Δ rad24-Δ* strains gave rise to equal sized (although small) colonies (Figure 8C). However, at higher temperatures, the difference between these two sets of strains became more obvious (Figure S6). This reveals a complex genetic interaction between the cold-sensitive growth properties of the suppressor strain (*cgi1121-Δ*) and the temperature-enhanced phenotypes of the *cdc13-Δ rad24-Δ* strain, as four genetic factors contributed to the growth characteristics of the *cdc13-Δ cgi121-Δ rad24-Δ* strain at different temperatures (loss of Cdc13 function, loss of Cgi121 function, the Tmp<sup>-</sup> phenotype and the cold-sensitive activity). Thus, whether the enhanced growth properties of the *cdc13-Δ cgi121-Δ rad24-Δ* strain is due to a genetic relationship between *CDC13* and *CGI121* cannot be determined.

**DISCUSSION**

The results presented here, as well as in several prior studies, establish that there is an additional phenotypic consequence at telomeres when cells are grown at higher temperatures, particularly above 34°. This phenotype can be observed even in wild type yeast and also contributes to the severity of phenotypes displayed by yeast strains bearing null mutations in the Ku heterodimer (FELDMANN and WINNACKER 1993; BOULTON and JACKSON 1996; FELDMANN et al. 1996), telomerase (Figure 2) and the t-RPA complex (Figure 3). We propose that the enhanced phenotype at higher temperatures is a synthetic genetic effect, as illustrated schematically in Figure 9, due to a mutation in a telomere-related complex combined with an additional defect which becomes particularly apparent at 34° to 36°.

This temperature-dependent defect also complicates the analysis of missense mutations in subunits of each of these complexes, particularly for the essential genes *CDC13, STN1* and *TEN1*, which suggests that a number of mutations in these three genes have been incorrectly categorized as temperature-sensitive alleles and are instead hypomorphic alleles. In particular, several observations
argue against the long-standing assumption that \textit{cdc13-1} encodes a conditional mutation. Even at 23°, \textit{cdc13-1} cells have reduced protein levels (Figure 4A) and thus presumably reduced \textit{CDC13} activity, which is consistent with the synthetic lethality even at 23° that occurs when \textit{cdc13-1} is combined with other telomere-specific mutations (\cite{Nugent1996, Polotnianka1998}). Furthermore, Cdc13-1 protein levels (Figure 4A) as well as the ability of the mutant protein to associate with telomeres (\cite{Vodenicharov2006}) are unchanged following a shift to 36°. Given that the defect encoded by \textit{cdc13-1} is so substantial that it is barely compatible with viability at 23°, we suggest that the combination of the \textit{cdc13-1} mutation with the defect that gives rise to the Tmp\textsuperscript{−} phenotype is responsible for inviability at 25° - 26°. Although the Tmp\textsuperscript{−} phenotype is most obvious at 34° to 36° (where it affects the viability of telomerase- and Ku-defective strains), telomere length in wild type cells is slightly reduced even at 30° relative to 23°. Furthermore, in \textit{yku80-Δ} cells, the robust DNA damage response that occurs at 37° (as measured by autophosphorylation of Rad53) can also be detected at 30° although to a lesser degree (\cite{Teo2001}). These observations are consistent with the idea that the Tmp\textsuperscript{−} phenotype can confer a synthetic defect even at lower temperatures (as illustrated in Figure 9) in the presence of a severe mutation such as \textit{cdc13-1}.

The Tmp\textsuperscript{−} phenotype is potentially the basis for incorrectly assigning ts properties to hypomorphic mutations in \textit{STN1} and \textit{TEN1} as well. In the extensive panel of \textit{stn1-} missense mutations reported here, every \textit{stn1-} strain which exhibited a telomere length defect at 23° (corresponding to mutations in 24 amino acids) was accompanied by reduced viability at 34° to 36°, a characteristic also displayed by the previously reported \textit{stn1-13} and \textit{stn1-63} mutant strains. This behavior is also similar to a recently reported panel of \textit{ten1-} mutations, which confer extremely elongated telomeres at permissive temperature, with growth impaired at 36° but not at lower temperatures (\cite{Xu2009}). We suggest that these \textit{ten1} alleles are also hypomorphs, rather than mutations that confer thermolabile Ten1 function. This suggestion also extends to a \textit{stn1-td} degron construct which retains a significant degree
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of viability at 37° after switching on the degron (Vodenicharov and Wellinger 2006). Collectively, we propose that there are currently no bona fide stn1-ts or ten1-ts reagents that are wild type at permissive temperature and depleted for the essential function encoded by STN1 or TEN1 following a temperature shift.

**Implications for analysis of telomere function at 36°.** Although we were unsuccessful in our attempts to identify conditional lethal mutations in STN1, we did recover five new cdc13-ts alleles with non-permissive temperatures between 30° and 33°. Unlike cdc13-1, these five mutations encode Cdc13 proteins that retain wild type, or near wild type, protein levels at 23° but are reduced by more than 10-fold at 36°. All five mutations map to the DNA binding domain (Mitton-Fry et al. 2002), with three residues (S531, N609 and S611) in close physical proximity (data not shown). This suggests a region of the DBD that might be particularly prone to thermo-sensitive perturbations (alternatively, the recovery of mutations in these three residues is simply a reflection of some bias in how the mutagenesis screens were conducted).

The identification of these new cdc13-ts strain allowed us to re-investigate a prior observation about the viability of Cdc13-depleted strains at non-permissive temperatures. Previous experiments, which have monitored the viability of cdc13-1 vs. cdc13-1 rad9-Δ strains following prolonged incubation at 36° (Weinert and Hartwell 1993; Lydall and Weinert 1995) have concluded that loss of Cdc13 function creates structure(s) which if left unrepaired, are lethal in the absence of Rad9 function (presumably as a consequence of unchecked cell cycle progression). We re-examined this observation using a newly generated cdc13-ts allele (cdc13-S611L) which was non-permissive for growth at 30° (Figure 5), which allowed us to compare the loss of viability in the absence of RAD9 at 32°, 34° and 36°. Even though 32° was fully non-permissive for CDC13 function, loss of viability was minimal. However, elevating the temperature to 34° or 36° resulted in a substantial further reduction in viability. Collectively, these observations suggest that even in the absence of RAD9, loss of CDC13
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function does not lead to inviability *per se*. Instead, we propose that lethality is the consequence of an additional defect occurring at 34° to 36°, when combined with *rad9-Δ* and *cdc13* depletion. If this proposal is correct, this suggests that hypotheses about the role of Cdc13 at telomeres as well as models derived from *cdc13-1*-induced DNA damage based on observations made at 34° to 36° may need to be re-visited, as the resulting phenotypes may be due to a more intricate set of genetic interactions than simply depletion of Cdc13. This point is further illustrated by the complex epistatic interaction that we observed between *cgi121-Δ* and *cdc13-Δ* mutations at different temperatures (Figure 8).

These results also have implications for genome-wide suppression and enhancer screens which have monitored viability of *cdc13-1* and *yku70-Δ* strains at temperatures ranging from 36° to 37.5° (Downey *et al.* 2006; Addinall *et al.* 2008; Addinall *et al.* 2011). At least some subset of genes recovered from these screens are presumably due to a genetic interaction with the Tmp phenotype, rather than with either *CDC13* or *YKU70*. Although this does not negate the importance of these studies, the potential for such genes to further our understanding of telomere biology will require a fuller understanding of what telomeric process(es) are impaired by elevated temperature.

In addition, the impairment due to the reduction in Cdc13-1 protein levels suggests that a subset of genes recovered from such screens are involved in protein stability and/or turn-over, rather than telomere biology. This would explain how *STM1*, which modulates translation by regulating formation of the 80S subunit of ribosomes (Balagopal and Parker 2011), functions as a high copy suppressor of *cdc13-1* (Hayashi and Murakami 2002). Similarly, the recovery of *san1-Δ* as a robust suppressor of *cdc13-1* (Downey *et al.* 2006; Addinall *et al.* 2008) is consistent with a role for San1 in mediating degradation of misfolded nuclear proteins (Fredrickson *et al.* 2011). More generally, *cdc13-1* suppressors which are involved ribosome function, protein degradation, RNA processing, protein transport and/or biosynthesis may have little direct relationship to telomere function.
What is the molecular basis for the Tmp$^-$ phenotype? This phenotype appears to be the consequence of a telomere-specific activity which is naturally temperature-labile even in wild type cells, as evidenced by telomere shortening at higher temperatures. This defect is presumably also responsible for the inviability observed in yku70-Δ and yku80-Δ strains, the enhanced senescence of telomerase-null strains and the reduced microcolony growth of RAD24-deficient cdc13-Δ, stn1-Δ and ten1-Δ null strains at 36°. Consistent with the premise that a common defect is responsible, the appearance of inviability in Ku-depleted cells exhibits phenotype lag (BARNES and RIO 1997). Similarly, the additive effect of temperature on telomerase-defective strains becomes more pronounced in later generations (Figure 2).

The temperature-induced inviability that occurs in yku70-Δ and yku80-Δ strains propagated at 36° has been characterized in detail by multiple laboratories, although with somewhat differing conclusions as to the molecular basis for the causative lesion (FELLERHOFF et al. 2000; TEO and JACKSON 2001; GRAVEL and WELLINGER 2002; MARINGELE and LYDALL 2002; SMITH et al. 2008). Several studies have lent support to the idea that an altered terminal DNA structure, which is generated when telomeres fall below a minimal length, is responsible. The shortening rate at 36° in Ku-depleted cells is inconsistent with a telomerase defect (GRAVEL and WELLINGER 2002), which is also supported by our results indicating that the temperature-dependent reduction in telomere length is not due to an impaired ability of telomerase at 36°. These observations therefore argue for an active shortening mechanism which occurs at high temperature. In the absence of Ku function, telomere-proximal regions replicate early (COSGROVE et al. 2002; LIAN et al. 2011). If high temperature further exacerbates this altered replication timing profile (for example, as a consequence of the shorter cell cycle time at 36°), we suggest that this could lead to incomplete replication of the duplex telomeric DNA tract by the conventional DNA replication machinery and hence telomere shortening.

Regardless of the molecular defect that underlies the Tmp$^-$ phenotype, this telomere-specific response is just one aspect of a pleiotrophic set of responses by cells to higher temperatures. Cells have
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a well-orchestrated mechanism for response to temperature fluctuations. Thus, other cellular pathways may also experience an analogous version of the Tmp– phenotype. The results described here also raise a cautionary note about using phenotypic analysis as the sole basis for categorizing ts mutations. This suggests that efforts to create genome-wide epistasis maps using a comprehensive array of temperature-sensitive reagents may need to take this into account (Ben-Aroya et al. 2008; Li et al. 2011).

ACKNOWLEDGEMENTS

We thank Madeleine Jennewein and Monika Walterscheid who each contributed to the initial stages of either of the two forward mutagenesis cdc13-ts screens, Edward K. Mandell for conducting the Evolutionary Trace analysis on Cdc13 and other members of the Lundblad lab for many helpful discussions during the course of this work. This research was supported by grant GM55867 from the US National Institutes of Health and by funding from the F.M. Kirby Foundation, the G. Harold and Leila Y. Mathers Charitable Foundation and the Chapman Foundation.
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LITERATURE CITED


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Gao, H., T. B. Toro, M. Paschini, B. Braunstein-Ballew, R. B. Cervantes et al., 2010 Telomerase recruitment in *Saccharomyces cerevisiae* is not dependent on Tel1-mediated phosphorylation of Cdc13. Genetics **186**: 1147-1159.


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VODENICHAROV, M. D., and R. J. WELLINGER, 2006 DNA degradation at unprotected telomeres in yeast is regulated by the CDK1 (CDC28/Clb) cell cycle kinase. Mol. Cell 24: 127-137.


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FIGURE LEGENDS

**FIGURE 1.**— Telomere length in wild type *S. cerevisiae* is temperature-sensitive. (A) Each haploid strain was propagated for ~100 generations by successive serial dilution of duplicate cultures at the indicated temperatures and genomic DNA prepped for telomere length analysis; two exposures were used to assemble the image to ensure equivalent signal intensity for each lane (as evaluated by comparison of non-telomeric bands, indicated by arrowheads). (B). The same experiment as in (A), with samples from 90 and 100 generation cultures grown at 36° displayed immediately adjacent to a sample grown at 23°.

**FIGURE 2.**— (A) Histogram displaying the growth characteristics of 42 *tlc1-* isolates grown at 23° vs. 36° for three successive streak-outs, corresponding to ~25, ~50 and ~75 generations of growth; isolates were scored for six phenotypic categories, ranging from a scale of 1 (severe senescence) to 6 (comparable to wild type growth), and a student’s t-test was used to assess the statistical significance between the two temperatures, as described previously (GAO et al. 2010). (B) Averaged phenotypic scores for the 42 *tlc1-* isolates propagated at 30°, 34° or 36° normalized to growth at 23°, with a negative value indicating enhanced senescence at a given time point, relative to the behavior of the same set of isolates at 23°.

**FIGURE 3.**— Microcolony growth of *cdc13-* *rad24-* and *stn1-* *rad24-* strains is temperature-sensitive. (A) Isogenic diploid strains bearing null mutations of the indicated genotype were dissected and germinated spores were photographed after growth was complete (48 hours at 30°, 32°, 34° and 36° or 72 hours at 23°, 26° and 28°) with a Zeiss Axioskop 50 with a Nikon Digital Sight DS-5M camera;
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multiple isolates were examined for each genotype, and representative examples are shown. The grid on
the \( cdc13-\Delta \) image corresponds to 62.5 x 62.5 \( \mu \text{m} \); all images were at the same magnification. (B) Photographs of \( cdc13-\Delta \ rad24-\Delta \) microcolonies from germinated spores generated by dissection of
\( cdc13-\Delta/CDC13 \ rad24-\Delta/RAD24 \) strain, following incubation for 3 days at the indicated temperature.
(C) Each \( cdc13-\Delta \ rad24-\Delta \) microcolony image shown in (B) was selected and the sum of the pixels in
the selected area was quantitated using Photoshop. The maximum and minimum value were eliminated
from each temperature group; mean and standard variation are shown. (D) Quantitation of \( stn1-\Delta \)
\( rad24-\Delta \) microcolonies from a \( stn1-\Delta/STN1 \ rad24-\Delta/RAD24 \) strain, processed as described in (B) and
(C). The \( stn1-\Delta \ rad24-\Delta \) microcolonies were \( \sim \) 2- to 3-fold smaller than \( cdc13-\Delta \ rad24-\Delta \)
microcolonies, although the experiments shown in (C) and (D) were performed separately, which
precluded a more quantitative comparison.

**Figure 4.**— The \( cdc13-1 \) and \( stn1-63 \) mutations behave like hypomorphic alleles. (A) Steady state
protein levels of the wild type Cdc13-(myc)\textsubscript{18} and mutant Cdc13-1-(myc)\textsubscript{18} proteins, expressed from a
\( CEN \) vector; extracts were prepared from strains grown at 23° or at 36° for 3.5 hours and analyzed by
anti-myc western on 8% SDS-PAGE. The strain also expresses an Est1-(myc)\textsubscript{13} protein (as an
integrated tagged construct), which provided an internal control for protein levels. (B) Comparison of
\( stn1-\Delta/pCEN \ STN1 \ vs. \ stn1-\Delta/pCEN \ stn1-63 \), as assessed by single colony propagation on rich media
(left panel) or cell cycle progression of liquid cultures (right panel) grown at 23° and 36°; arrows
indicate that cell division still occurs at 36°. (C) Viability of \( RAD24 \) and \( rad24-\Delta \) versions of the \( stn1-\Delta \)
\( pCEN \ stn1-63 \) strain at the indicated temperatures; serial dilutions were plated on pre-warmed rich
media plates and photographed after 2.5 days (for \( \geq 28^\circ \) incubations) or 4 days (for 23° and 25°). (D)
Steady state protein levels of the Stn1-63-(myc)\textsubscript{7} protein, expressed from a \( CEN \) vector, assessed as

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described in Part (A). (E) Telomere length of the \textit{stn1-}\textDelta/p\textit{CEN stn1-63} strain compared to the isogenic \textit{stn1-}\textDelta/p\textit{CEN STN1} strain at 23°.

\textbf{FIGURE 5.—} Characterization of new \textit{cdc13-ts} mutations. (A) Steady state protein levels of mutant Cdc13-(myc)\textsubscript{18} proteins compared to wild type Cdc13-(myc)\textsubscript{18}, assessed as in Figure 4A. (B) Viability of the indicated strains, with \textit{cdc13-ts} mutations integrated into the genome, assessed as in Figure 4C.

\textbf{FIGURE 6.—} Temperature-dependent effects on viability of a \textit{cdc13-S611L rad9-}\textDelta strain. (A) Mid-log cultures grown at 23° of \textit{cdc13-S611L} (left panel) or \textit{cdc13-S611L rad9-}\textDelta (right panel, duplicate samples) strains were shifted to the indicated temperatures and incubated for up to 8 hours; viability was determined by plating appropriate dilutions on rich media plates which were incubated at 23° for three days. Three independent repetitions of this experiment produced essentially identical results. (B) Flow cytometry profile of log-phase cultures of the indicated strains from the experiment shown in (A), fixed and stained with SYTOX green.

\textbf{FIGURE 7.—} Characterization of \textit{stn1-I73S} and \textit{stn1-I73A} strains. (A) Photo-micrographs of germinated \textit{stn1-I73S} and \textit{stn1-I73S rad24-}\textDelta spores. (B) Single colony streak-outs of isogenic \textit{stn1-I73A} and \textit{STN1} haploid strains (generated by dissection of a \textit{stn1-I73A/STN1} diploid), incubated at 23°, 30° and 36°. (C) Telomere length analysis of \textit{stn1-I73A} and \textit{STN1} isolates from part (B), after \textasciitilde40 generations of growth at 23° or 30°.

\textbf{FIGURE 8.—} Loss of \textit{CGI121} function bypasses null mutations in \textit{CDC13} and telomerase. (A) Comparison of colony sizes of \textit{CGI121} and \textit{cgi121-}\textDelta isogenic strains, propagated at the indicated temperatures. (B) Comparison of the senescence phenotypes of 26 \textit{tlc1-}\textDelta \textit{CGI121} isolates with 22 \textit{tlc1-}
Δ \textit{cgi121}Δ isolates at 32° and 36°, analyzed as in Figure 2B. (C) Three colonies each of \textit{cdc13}Δ \textit{cgi121}Δ \textit{rad24}Δ and \textit{CDC13} \textit{cgi121}Δ \textit{rad24}Δ generated following dissection of the appropriate heterozygous diploid strain at 23°; see Figure S6 for the relative growth of these two strains at 30° and 36°.

\textbf{FIGURE 9.}—A schematic depiction of the additive contribution of the Tmp− phenotype to the viability of strains bearing mutations in telomere-related proteins, based on a decline in “telomere function” which occurs with increasing temperature. Since the molecular basis for the Tmp− phenotype is unknown, “telomere function” is defined as the collection of activities that maintain chromosome ends as fully replicated and capped telomeres.
**TABLE 1.** Strains used in this study.

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* additional genotype: ura3-52/ura3-52 lys2-801/lys2-801 trp1-Δ1/trp1-Δ1 his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1

** additional genotype: bar1-Δ::cNAT ura3-52 lys2-801 trp-Δ1 his3-Δ200 leu2-Δ1
Temperature impairment of telomere function

**TABLE 2.** Plasmids used in this study.

<table>
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<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Vector backbone</th>
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<td>YCplac111</td>
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The panel of temperature-sensitive missense mutations introduced into the *CDC13* gene:

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<td><em>cdc13-N609A</em></td>
<td>pVL3956</td>
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<tr>
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Figure 5

A

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<td>0.04</td>
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B

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