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13. ABSTRACT (Maximum 200 words) In the yeast <i>S. cerevisiae</i> , <i>CDC13</i> has been shown to be important for both telomere replication and maintenance of chromosome integrity. One model for Cdc13p function is that it associates with telomeres as a single-stranded binding protein and facilitates complete replication of the telomeric DNA by protecting the chromosome end and regulating access of telomerase to the chromosome terminus. Additional genes required for telomere capping activity and length maintenance were characterized through examination of the relationship between genes required for repair of DNA double strand breaks, <i>CDC13</i> , and genes encoding telomerase components. These data suggest that the Ku heterodimer, Cdc13p, and telomerase participate in distinct pathways required for telomere function. To further explore the mechanism of telomere replication, I identified <i>CDC13</i> and <i>EST2</i> interacting proteins via yeast two-hybrid screens. Characterization of the <i>EST2</i> interacting proteins may reveal additional telomerase components or regulators. <i>STN1</i> , a high-copy suppressor of <i>cdc13-1^{ts}</i> , was identified as a <i>CDC13</i> -interacting protein. Interestingly, the Stn1p-Cdc13p two-hybrid interaction is abolished by the <i>cdc13-2^{est}</i> mutation. Thus, the <i>cdc13-2^{est}</i> allele may define a domain of interaction with Stn1p that is required for positive regulation of telomere replication.			
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FOREWORD

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RESEARCH SUMMARY

INTRODUCTION

Subject

Telomeres, the physical ends of chromosomes, have long been observed to be functionally distinct from those ends generated by chromosomal breakage. Whereas DNA double strand breaks are subject to fusion with other ends, telomeres insulate chromosomes from such end-to-end fusion events (1). Telomere length in many immortal eukaryotic cell populations is maintained at least in part through the action of telomerase, a reverse transcriptase that extends the terminal GT-rich telomeric DNA strand. However, telomerase alone is not responsible for maintaining the length and integrity of chromosome ends. One factor proposed to contribute to telomere end protection in yeast is the single-strand telomere DNA binding protein, Cdc13p (2, 3), as the loss of *CDC13* activity results in rapid degradation of the C-rich strand of the telomere and a consequent disruption of telomere integrity (4). In a large mutant screen to identify yeast mutants defective for telomere replication, our lab identified a novel allele of *CDC13*, an essential gene that has been implicated in maintaining telomere integrity (2, 4). This mutant allele, *cdc13-2^{est}*, has a phenotype virtually identical to that displayed by a telomerase-minus strain. However, enzyme levels are normal in extracts prepared from a *cdc13-2^{est}* strain, showing that this mutation defines a function of *CDC13* that is required *in vivo* for telomere replication but not *in vitro* for enzyme activity. Both genetic and biochemical data led us to propose that Cdc13p is a single-stranded telomere binding protein required both to protect the end of the chromosome and to regulate access of telomerase to the chromosomal terminus (2). The goal of my research is to identify genes that are involved with *CDC13* in mediating telomere function, and to determine through analysis of these genes how telomere replication is regulated in yeast.

Background

Telomeres, specialized structures at the ends of chromosomes, help maintain the stability of the genome and are essential for continued cell proliferation. Telomeres are usually composed an array of simple tandem DNA repeats, complexed into a non-nucleosomal chromatin structure. The DNA repeats typically consist of a short G/C-rich motif, with the 3' end of the G-rich strand extending as a single-strand beyond the duplexed region (reviewed in (5)). In *S. cerevisiae*, the telomeric tract extends for approximately 300-500 base pairs and the consensus repeat sequence is G₂₋₃T(GT)₁₋₆, abbreviated G₁₋₃T. The enzymes that replicate the genome are not capable of fully duplicating the ends of the chromosomes; thus a special mechanism is required to maintain these sequences through replicative cycles. Most eukaryotic cells employ telomerase, a telomere-specific DNA polymerase containing an RNA template, to extend telomeric DNA sequences and allow cells to maintain telomere length through cycles of cell division. Telomerase extends the G-rich DNA strand by adding a short sequence that is determined by the template sequence of the telomerase RNA (reviewed in (6)).

A few years ago it was determined that the RNA subunit of telomerase in *S. cerevisiae* is encoded by *TLC1* (7). Recently, through sequence comparison with the telomerase catalytic

subunit from *Euplotes*, the catalytic subunit of *Saccharomyces cerevisiae* telomerase was determined to be encoded by *EST2* (8, 9). Thus, the core constituents of *S. cerevisiae* telomerase have been identified. In *S. cerevisiae*, telomere replication has been shown to depend not only upon the core subunits of telomerase, but also on *EST1*, *EST3* and *CDC13* (10, 11). Although only mutations in *EST2* or *TLC1* result in the loss of telomerase activity *in vitro* (8, 9, 12, 13), mutations in all five genes result in eventual senescence, whereby the telomeric repeat tract becomes progressively shorter until telomere function is lost, resulting in chromosome loss and cell death.

BODY

I. Analysis Of Mutants That Display A Synthetic Phenotype With *Cdc13-1^{ts}*

I have observed that elimination of the telomerase RNA template in a *cdc13-1^{ts}* strain results in a lowered maximum permissive temperature for growth as well as an exaggerated senescence phenotype (2). This synthetic phenotype with *cdc13-1^{ts}* has also been observed with the other genes (*EST1*, *EST2*, *EST3*) identified in the telomere replication pathway. Thus, the synthetic phenotype appears to be a general consequence of loss of the *EST/TLC1* pathway in a *cdc13-1^{ts}* background. As a means to identify additional genes in the telomere replication pathway, I had proposed a screen to identify mutations that not only displayed a similar synthetic phenotype with *cdc13-1^{ts}* but also displayed telomere length alterations. I had expected that such a screen should yield not only components of telomerase, but also other genes that regulate telomere length. Before I undertook the screen, lab member Lyle Ross discovered that mutations in *yku80* enhanced the senescence of an *est1-Δ* mutant strain. This data suggested that the short telomere length phenotype previously observed in *yku80-Δ* cells is not a simple consequence of compromised telomerase activity, and prompted me to explore the possibility *YKU80* functions with *CDC13* in maintaining telomere integrity.

yKU80 encodes a protein that, as a heterodimer with Hdf1p, is capable of binding double strand DNA ends or other discontinuities in DNA structure (14, 15). The Ku protein heterodimer, a complex comprising Rad50/Mre11/Xrs2, and the Sir2p, Sir3p and Sir4p heterochromatin associated proteins have been shown to be critical for non-homologous DNA double-strand break repair in yeast (14, 16, 17, 18). Strains bearing mutations in *HDF1*, *yKU80*, and *RAD50* have all been shown to exhibit shortened telomeres (17, 19, 20). The role of these genes in telomere replication or length maintenance has been unclear, although very recent data has shown that Ku80p can be found specifically associated with telomeric DNA *in vivo* (21). In order to explore the role of these genes in relation to telomeres, I undertook a genetic analysis of strains deficient for *HDF1*, *yKU80*, *RAD50* or *MRE11*. These experiments led to a publication in Current Opinion in Biology ((22) Appendix A.)

Methods for analysis of synthetic mutants

Yeast Strains

The *HDF1*, *YKU80*, *RAD50*, *MRE11* genes were disrupted in diploids using fragments generated from PCR amplification of the kanMX2 cassette (23). Primer pairs for each gene were

designed with 46 base pairs of homology to regions at the start and stop codons of the open reading frame. A disruption of *MRE11* in a haploid was also created using a fragment from pKJ1112-S (provided by J. Haber), in which the coding region was replaced with *URA3*. *SIR3* was disrupted in a diploid with a *LEU2* cassette from pLP47 (kindly provided by L. Pillus). Standard genetic techniques were used to create the various double mutant combinations.

In the haploid UCC3505 strain used for the silencing studies (7), *TLC1* was replaced with the *LEU2* gene as in Lendvay et al. 1996 and *SIR3* was disrupted with the *sir3Δ::LEU2* fragment from pLP47. *EST1*, *EST2*, *MRE11*, *RAD50*, and *HDF1* were knocked-out with the kanMX2 cassette, as described above. The mutant *cdc13-1^{ts}* and *cdc13-2^{est}* alleles of *CDC13* were integrated into the UCC3505 strain via plasmids pVL501 and pVL503, which contain *TRP1* and *LYS2* as selectable markers; the wild-type gene was popped out using α -amino-adipate (ICN) as a counter-selection.

Genetic Manipulations

All strains were grown in standard selective or rich media, and manipulated at temperatures $\leq 23^{\circ}\text{C}$ unless otherwise indicated. Yeast transformation was performed by the lithium acetate method. In experiments involving serial 10-fold dilutions of cells, initial cell density was determined using a hemocytometer and approximately equivalent numbers of cells were placed in microtiter dishes. Senescence was monitored by streaking for independent colonies multiple times in succession or by analyzing the percent of viable colony forming units after serially passaging the strains in liquid cultures. For analysis of high-copy suppression of ku temperature sensitivity, at least two transformants from a minimum of two independent transformations were examined for each plasmid/ strain combination.

DNA preparation and southern

Yeast genomic DNA preps and southern blots to monitor telomere length were performed as previously described (11).

Results & Discussion: Synthetic Mutant Analysis

Genetic analysis

As described below, epistasis analysis revealed that genes encoding telomerase components, the Ku complex and Cdc13p each appear to contribute separate roles required for telomere replication and cell viability. Any combination of Ku mutations with telomerase mutations resulted in an exacerbated phenotype; strains with deletions in *EST1*, *EST2* or *TLC1* exhibited accelerated inviability when they were also lacking either *HDF1* or *yKU80* (Fig.1A). Haploid *hdf1-Δ est2-Δ* and *yku80-Δ est2-Δ* double mutant spores generated from the appropriate heterozygous diploid strain gave rise to colonies on the dissection plate that consisted of mostly inviable cells, as evidenced by the fact that such colonies could not be further propagated, whereas *est2-Δ⁻* mutant spores handled in parallel exhibited an initial growth phenotype comparable to that of wild type (Fig. 1A), with no substantial increase in cell death observed until 50 to 75 generations later. The simplest explanation for this synthetic near-lethality is that increased telomere shortening occurs due to different mutations contributing to two separate telomere length maintenance defects. Similarly, combining *hdf1* or *yku80* mutations with the *cdc13-1^{ts}* mutation resulted in impaired growth, in that the maximal permissive temperature of

hdf1-Δ cdc13-1^{ts} and *yku80-Δ cdc13-1^{ts}* double mutant strains is reduced, relative to the single *cdc13-1^{ts}* strain (Fig. 1B). Even at permissive temperature, these double mutant strains have a noticeable growth defect (Fig. 1B). This synthetic phenotype does not result simply from the loss of a DNA repair pathway required to repair damage generated in the *cdc13-1^{ts}* strain, since a *sir3-Δ* mutation, which also eliminates the same DNA end-joining repair pathway, does not enhance the defect of a *cdc13-1^{ts}* mutation.

Although the Ku complex and Rad50/Mre11/Xrs2 are both required for efficient repair of DNA double-strand breaks, these genes can be placed in separate genetic pathways with respect to telomere function. In contrast to the observations with *est-Δ ku-Δ* double mutants; *mre11-Δ* and *rad50-Δ* fail to enhance the telomere length or cell viability defects of telomerase mutants (Fig. 2A). Thus, in contrast to the Ku complex genes, both *MRE11* and *RAD50* appear to act in the telomerase mediated pathway for telomere replication. Consistent with this epistasis placement, *mre11-Δ* and *rad50-Δ* mutations exhibit enhanced growth defects with either *cdc13-1^{ts}* mutations or *ku* mutations (Fig. 2B).

Silencing assay to assess state of telomeric chromatin

The telomere-specific protection that distinguishes natural chromosomal termini from double strand breaks is presumably mediated through formation of a specific telomere chromatin structure. It has been thought that telomerase activity is not required to mediate the protection from end-to-end fusions that telomeres provide, as chromosomes in mammalian cells lacking telomerase activity are not necessarily prone to end-recombination. Thus, one potential role for the Ku heterodimer could be to prevent chromosome ends from fusing, potentially as a structural component of telomeric chromatin. One method to probe the integrity of telomeric chromatin is to assess the state of expression of reporter genes placed in subtelomeric regions (1). The degree of transcriptional repression (called telomeric position effect, or TPE) has been interpreted as a reflection of the integrity of telomeric chromatin. Fig. 3 shows that TPE is substantially altered by the loss of Ku activity but is unaffected in strains carrying deletions of the *MRE11* or *RAD50* genes. Expression of a telomere-located *URA3* gene can be monitored by assessing either growth in the absence of uracil or growth in the presence of a drug inhibitory to *Ura⁺* cells (7).

Elimination of Ku function resulted in an intermediate effect on the expression of a *URA3* reporter gene at temperatures permissive for long term growth of this Ku-defective strain. However, when TPE in an *hdf1* strain was examined immediately after transfer to 36° (when the strain was still viable), repression of a telomere-located *URA3* reporter gene was completely abolished, comparable to that observed when an essential component of telomeric chromatin, Sir3p, is deleted. In contrast, TPE was unchanged in strains defective for the telomerase epistasis group; *mre11-Δ*, *rad50-Δ*, *est1-Δ*, *est2-Δ*, *tlc1-Δ* or *cdc13-2^{est}* mutant strains showed no differences relative to wild type (Fig. 3). Thus, the Ku complex appears to play a crucial role in maintaining telomeric chromatin structure. At high temperatures its function is essential for maintaining transcriptional repression, whereas at low temperature the slower cell cycle kinetics or another activity compensate for its absence.

Over-expression of telomerase components suppresses ku- temperature sensitivity

Previous work has shown that cells devoid of Ku function show a temperature sensitive growth phenotype with a phenotypic lag, whereby cells proliferate for at least 20 generations

before they die (24). This temperature-sensitive phenotype of *hdf1* or *yku80* mutants could be suppressed by over-expression of *EST1*, *EST2* or *TLC1* (Fig. 4), further supporting the genetic interaction between telomerase and the Ku complex. Over-expression of *EST1*, *EST2* or *TLC1* could also at least partially suppress the temperature sensitive silencing defect of *hdf1*- Δ cells. The simplest interpretation of the delayed inviability of *hdf1* and *yku80* strains is that at low temperature, other pathways for maintaining telomere function compensate for loss of Ku protein at telomeres, but these other pathway(s) become inadequate at high temperatures, potentially for kinetic or stability reasons.

Conclusions

The Ku complex plays a critical role at telomeres that is distinct from that of either telomerase or Cdc13p, and affects the maintenance of telomeric chromatin structure. It has been recently observed that loss of either *YKU80* or *HDF1* results in altered telomere end structure, such that there appears to be extensive terminal single-stranded G₁₋₃T DNA present throughout the cell cycle (21). Possible models for the function of the Ku complex at telomeres include a role in protecting chromosome ends from degradation or deleterious recombination events, such as end-to-end fusions. Alternatively, the complex could be required for mediating a higher order chromatin architecture that is critical for complete synthesis of lagging strand DNA at telomeres. The roles of Rad50p and Mre11p in telomere function are not clear; the genetic analysis cannot illuminate their biochemical function. It is possible that these genes are responsible for processing or regulating the DNA end structure such that telomerase can efficiently utilize its substrate at the proper time. The activities of these genes may have been co-opted by telomeres to function at the chromosome end in a manner similar to their roles in double strand break repair, although the critical difference is that telomeres do not normally allow end-to-end joining with other chromosome ends.

II. Identification Of Telomerase Components And Cdc13p Associated Proteins.

I had proposed to find additional telomerase components by either isolating mutations that exacerbate the temperature sensitivity of *cdc13-1^{ts}* and alter telomere length or by identifying gene products that interact with *CDC13* in a two-hybrid screen. With the recent discovery that *EST2* encodes a catalytic component of the telomerase enzyme (8, 9), I undertook a yeast two-hybrid screen to identify *EST2*-interacting proteins as a more direct route to the identification of additional telomerase components. I also expected to identify potential telomerase regulators or other proteins critical for telomere replication. Nathan Walcott, a technician in the lab, provided technical assistance in this screen. In addition to the *EST2* two-hybrid screen, I also undertook the *CDC13* two hybrid screen in order to identify proteins critical to *CDC13* function. One interpretation of the *cdc13-2^{est}* mutant phenotype is that the mutation renders the Cdc13-2^{est} protein unable to interact with another protein critical for telomere replication; my goal was to try to identify this interacting protein.

Methods for identification of EST2 or CDC13-interacting factors in two-hybrid.
Identification of interacting factors using the two-hybrid system.

The two hybrid system detects protein-protein interactions via *in vivo* reconstitution of the activity of a transcriptional activator, where one protein is fused to the DNA binding domain of either *GAL4* or LexA and a second protein or cDNA library is fused to a transcriptional activation domain (25, 26). Reconstitution of the transcriptional activator is detected by screening for transcription of a reporter gene that contains a *GAL4* or LexA binding domain in its promoter. A *GAL4* based system (27, 28) was used in screening for Est2p and Cdc13p interacting proteins. I initially screened *S. cerevisiae* libraries for Cdc13p interacting clones in strain backgrounds HF7c, Y190, and pJ69-4A, and determined that the latter strain was most optimal for my purposes. The pJ69-4A strain carries three reporter gene constructs, allowing selection for interacting clones by the ability to grow in the absence of histidine or adenine and to produce β -galactosidase activity (29).

Standard techniques for DNA transformation of *S. cerevisiae* were followed. Library transformations were initially plated on media lacking adenine. Colonies that arose were subsequently transferred to media lacking histidine (and supplemented with 2 mM 3-aminotriazole) and to media that only maintained selection for the bait and library plasmids for the β -galactosidase activity assay. The β -galactosidase activity was detected by overlaying a layer of agarose containing X-gal (30). The pAS1, pDAB1, and pACT2.2 plasmids and *S. cerevisiae* cDNA libraries used in these screens were generously provided to us by Steve Elledge.

Candidate interacting clones were put through two tests to detect false positives. First, each plasmid was tested for whether it activated transcription of the reporter genes in the absence of the Cdc13p or Est2p fusion. Second, the original phenotype was confirmed and the specificity of the interaction between the candidate plasmid (the prey) and the *CDC13*-fusion or *EST2*-fusion (the baits) was tested by assessing interaction of the candidate with the *GAL4-SNF1* fusion. Candidates passing these two controls were rescued from the yeast cells, transformed into bacteria and plasmid DNA preps were sequenced to determine the encoded protein.

Results & Discussion: Identification of Est2p and Cdc13p associated components.

The EST2 Screen

Prior to embarking on the *EST2* based screen, an *EST2*-fusion plasmid suitable for screening had already been determined by Melissa Sistrunk. The construct encodes the entire *EST2* protein, fused in frame at its amino-terminus with the Gal4 DNA binding domain and an HA-epitope tag. This construct complements the null *est2*- Δ phenotype and the fusion construct can be detected on a western gel.

A total of $\sim 1.8 \times 10^6$ library transformants were screened for interaction with *EST2*, yielding 20 different genes testing as potential Est2p-interactors (Table 1). I do not expect that all of the proteins encoded by these genes have functional interactions with Est2p. The genes identified in this screen are in a preliminary state of characterization; I will pursue only those that appear to be functionally relevant to telomere replication. I am initially constructing null mutations in the novel ORFs to determine if they are essential or have phenotypes suggestive of a role in telomere length regulation. The phenotypes I am initially characterizing include: altered telomere length (longer or shorter), a senescent growth, altered expression of telomere located genes (telomeric silencing), or altered telomerase activity as measured by an *in vitro* assay. If I

identify a gene that is essential for cell growth, in order to further study its function I will generate conditional alleles using strategies described in (31).

Two of the genes isolated in the screen, *CAC1/RLF2* and *SDS3*, have been shown through previous characterization by other labs to function in some aspect of telomere chromatin formation or maintenance (32, 33, 34, 35). It is not likely that either of these genes encode components of the telomerase holoenzyme; I will pursue the characterization of the relationship of these gene products to telomerase at a later point.

The CDC13 Screen

I have observed that over-expression of *CDC13* is toxic in wild-type yeast cells (Fig. 5). Thus, an important first step in the *CDC13* screen was to define suitable *CDC13* fusion constructs. First, I tested whether expressing the *CDC13* fusion construct from a centromeric, rather than 2 μ plasmid backbone would sufficiently reduce proteins levels; unfortunately, although the amount of Cdc13p may have been reduced in the cells, the toxicity was not sufficiently reduced for screening purposes. As partial gene fusions have successfully been used to identify interacting proteins in the two-hybrid system (36), I next constructed a series of *CDC13* deletions, summarized in Fig. 6. To try to assess the functional competence of these constructs, they were tested for their ability to complement the phenotypes of *cdc13- Δ* null, *cdc13-1^{ts}*, or *cdc13-2^{est}* cells. As all of the bait constructs were epitope tagged, the stability of the expressed protein was assessed by western blot. With the exception of the *cdc13-1^{ts}* mutant construct, any non-toxic construct that contained the *CDC13* DNA binding domain did not make sufficient protein to be detected in 100 μ g of extract. I choose to do the screen with pVL705, a well expressed, non-toxic construct that contains an in-frame deletion of the DNA binding domain. Initially, pVL587 was also used in the screen; this construct was abandoned when the polyclonal anti-Cdc13p antibody did not recognize the protein construct on a western, suggesting that the peptide produced may be misfolded.

A total of 2.3×10^6 library transformants were screened for interaction with pVL705. Two genes were identified as encoding potential Cdc13p-interactors, *STN1* and an uncharacterized open reading frame (ORF), YKL117w. Six different *STN1*-fusion plasmids were identified in the screen, all of which encoded at minimum the carboxyl-terminal half of Stn1p (Fig. 7).

Testing Cdc13p-interacting proteins for sensitivity to the cdc13-2^{est} mutation.

Based on the assumption that the *cdc13-2^{est}* mutation disrupts a critical protein-protein interaction necessary for telomerase regulation, I tested the strength of the interaction of the *STN1* and YKL117w library plasmids with the *cdc13-2^{est}* bait construct. Interestingly, while the *STN1* fusion constructs display a strong interaction with pVL705, they do not appear to interact with the *cdc13-2^{est}* bait construct. I have recently isolated additional alleles of *CDC13* with short telomere phenotypes; the interaction of these alleles will be tested with the *STN1* fusions. One caveat to these experiments is that while the *cdc13-2^{est}* bait construct produces a functional protein able to complement the essential phenotype of *cdc13- Δ* cells and recapitulates the phenotype of the genomic *cdc13-2^{est}* mutation, the protein is not present at the same level as the pVL705 construct. To definitively test whether the *cdc13-2^{est}* mutation disrupts an interaction

with Stn1p, I am integrating epitope tagged versions of *STN1*, *CDC13* and *cdc13-2^{est}* to determine if the proteins co-immunoprecipitate.

Directed Two-Hybrid: Testing known proteins for interaction.

Both Est2p and Cdc13p may interact with other proteins that are known to have telomere-related functions. The proteins that I have specifically tested for interaction with Cdc13p include Est1p, Est2p, Est3p, Sir4p and Rap1p (37, 38) (Table 2); these latter proteins are associated with telomeric chromatin. Since over-expression of full length *RAP1* is toxic (39), I used a previously characterized *RAP1* truncation construct (40) to assay for an interaction with Cdc13p. Although over-expression of *EST1* can suppress the senescence phenotype of the *cdc13-2^{est}* allele, it does not appear that Est1p directly interacts with Cdc13p, at least as assayed by two-hybrid. While genetic suppression of a mutant phenotype does not necessarily suggest a physical interaction, one caveat to the negative result obtained by two-hybrid is that the *EST1*-fusion construct used in the two-hybrid assay is not capable of suppressing *cdc13-2^{est}* senescence.

Many of the Est2p and Cdc13p interactors identified in the screens were also directly tested for interaction with this subset of telomere related genes. YKL117w, identified in the Cdc13p screen, was also observed to interact with Est2p, suggesting a possible physical or regulatory connection between Cdc13p and Est2p. Sds3p, isolated in the *EST2* screen, was observed to additionally interact with Est1p.

Characterization of F2 (YKL117w)

The second Cdc13p interacting protein identified in the two hybrid screen is an acidic 24.1 kD protein. The amino acid sequence of YKL117w is highly similar to a *S. pombe* gene and also has potential human, chicken, and *C. elegans* sequence homologs. The putative *H. sapiens* homolog has been observed to co-purify with Hsp90 and Hsp70; its function remains unknown (41). In order to determine if YKL117w has a significant function in telomere replication, I knocked out its ORF and analyzed the phenotypes of the null cells. At this point, there is no data to suggest that YKL117w is critical for telomere replication or length regulation. The gene is not essential, and null cells show no alteration in telomere length, cell growth, or telomeric silencing. In addition, overproduction of YKL117w does not suppress the senescence associated with loss of telomerase. I am in the process of determining if loss of YKL117w further impairs either telomerase deficient or *cdc13-1^{ts}* cells. The final experiments I am pursuing with this ORF are to test whether it is required for regulation of Cdc13p through the cell cycle.

Characterization of STN1

STN1 was originally identified in a screen for high copy suppressors of the temperature sensitivity of *cdc13-1^{ts}* cells (42). *STN1* is essential for viability, although its precise function is not known and its sequence reveals no recognizable motifs. The extremely long telomere phenotype of the *stn1-13* mutant (42) suggests that *STN1* is required for proper telomere length regulation. I observed that over-expression of certain *STN1*-fusion constructs results in elongated telomeres. The generation of these lengthened telomeres by over-production of these constructs is dependent on telomerase and *yKU80*, but not on *RAD52* or *TEL1*. Similarly, to maintain telomere length in *stn1-13* mutant cells, telomerase and *yKU80* are required (Fig. 8 and data not shown). Thus, *STN1* may function to negatively regulate telomere length, possibly

through negative regulation of telomerase activity. The high copy suppression and Cdc13p two-hybrid interaction data together suggest that Stn1p and Cdc13p may function as a complex. In order to understand the role of Cdc13p and Stn1p in telomere replication, my focus for the next year will be to address the following questions:

C. What is the nature of the interaction between Cdc13p and Stn1p?

My goal with these experiments is to determine if the mutant phenotype of *cdc13-2^{est}* or *stn1⁻* can be attributed (at least in part) to a loss of the ability to interact with the other protein.

- Determine if epitope tagged Cdc13p and Stn1p co-immunoprecipitate from yeast extracts.
- Determine if mutant versions of epitope tagged Cdc13p and Stn1p co-immunoprecipitate.

D. Are *CDC13* or *STN1* protein levels cell cycle regulated, and is such regulation critical for function?

E. Are either Cdc13p or Stn1p modified during the cell cycle, and if so, what is responsible for the modification?

4. Do Cdc13p or Stn1p localize to telomeres *in vivo*?

- Determine if Cdc13p or Stn1p are specifically associated with telomeric DNA *in vivo* using formaldehyde cross-linking.

5. In addition to Cdc13p, what does Stn1p physically interact with?

I have had very limited success in finding a *STN1* two-hybrid bait construct that does not auto-activate transcription. Therefore, two alternative approaches to identifying such proteins will be taken.

- Use the tagged *STN1* strain to look for interaction (by co-IP) with other proteins that function in telomere replication and maintenance.
- Identify high copy suppressors of temperature sensitive *stn1* alleles or of *stn1-13 cdc13-1^{ts}* double mutants.

Additional temperature sensitive alleles of *STN1* will be generated either through PCR mediated random mutagenesis or through alanine scanning mutagenesis.

CONCLUSIONS

Telomere length regulation may play a critical role in determining the proliferative potential of cells, indicating involvement in the related processes of aging and cancer. Data from the work I completed during the previous funding period suggests that multiple genetic pathways contribute to telomere function. In particular, my data, together with recent data from other labs, suggests that the Ku heterodimer plays an important role in protecting chromosome ends.

CDC13 and *STN1* appear to function in an independent pathway that contributes not only to maintaining telomere integrity, but also to telomere replication. Through characterization of proteins identified in the *EST2* and *CDC13* two-hybrid screens, I expect to learn more about the mechanism of telomere replication and the regulation of telomerase.

REFERENCES

1. V. A. Zakian, *Annual Review of Genetics* **30**, 141-172 (1996).
2. C. I. Nugent, T. R. Hughes, N. F. Lue, V. Lundblad, V. Lundblad, *Science* **274**, 249-252 (1996).
3. J. J. Lin, V. A. Zakian, *Proc. Natl. Acad. Sci. U S A* **93**, 13760-13765 (1996).
4. B. Garvik, M. Carson, L. Hartwell, *Mol. Cell. Biol.* **15**, 6128-6138 (1995).
5. E. H. Blackburn, C. W. Grieder, Eds., *Telomeres*. (Cold Spring Harbor Press, Cold Spring Harbor, New York, 1995).
6. C. I. Nugent, V. Lundblad, *Genes Dev.* **12**, 1073-1085 (1998).
7. M. S. Singer, D. E. Gottschling, *Science* **266**, 404-409 (1994).
8. J. Lingner, et al., *Science* **276**, 561-567 (1997).
9. C. M. Counter, M. Meyerson, E. N. Eaton, R. A. Weinberg, *Proc. Natl. Acad. Sci. U S A* **94**, 9202-9207 (1997).
10. V. Lundblad, J. W. Szostak, *Cell* **57**, 633-643 (1989).
11. T. S. Lendvay, D. K. Morris, J. Sah, B. Balasubramanian, V. Lundblad, *Genetics* **144**, 1399-1412 (1996).
12. J. Lingner, T. R. Cech, T. R. Hughes, V. Lundblad, *Proc. Natl. Acad. Sci. U S A* **94**, 11190-11195 (1997).
13. M. Cohn, E. H. Blackburn, *Science* **269**, 396-400 (1995).
14. G. T. Milne, S. Jin, K. B. Shannon, D. T. Weaver, *Mol. Cell. Biol.* **16**, 4189-4198 (1996).
15. M. R. Lieber, U. Grawunder, X. Wu, M. Yaneva, *Curr. Opin. Genet. Dev.* **7**, 99-104 (1997).
16. J. K. Moore, J. E. Haber, *Mol. Cell. Biol.* **16**, 2164-2173 (1996).
17. S. J. Boulton, S. P. Jackson, *EMBO J.* **17**, 1819-1828 (1998).
18. Y. Tsukamoto, J. Kato, H. Ikeda, *Nature* **388**, 900-903 (1997).
19. S. E. Porter, P. W. Greenwell, K. B. Ritchie, T. D. Petes, *Nucl. Acids Res.* **24**, 582-585 (1996).
20. K. M. Kironmai, K. Muniyappa, *Genes Cells* **2**, 443-455 (1997).
21. S. Gravel, M. Larrivee, P. Labrecque, R. J. Wellinger, *Science* **280**, 741-745 (1998).
22. C. I. Nugent, et al., *Curr. Biol.* **8**, 657-660 (1998).
23. A. Wach, A. Brachat, R. Pohlmann, P. Philippsen, *Yeast* **10**, 1793-1808 (1994).
24. G. Barnes, D. Rio, *Proc. Natl. Acad. Sci. USA* **94**, 867-872 (1997).
25. S. Fields, O. Song, *Nature* **340**, 245-246 (1989).
26. C. Chein, P. L. Bartel, R. Sternglanz, S. Fields, *Proc. Natl. Acad. Sci. USA* **88**, 9578-9582 (1991).
27. C. Bai, S. Elledge, *Methods Enzymol.* **273**, 331-347 (1996).
28. S. M. Hollenberg, R. Sternglanz, P. F. Cheng, H. Weintraub, *Mol. Cell. Biol.* **15**, 3813-3822 (1995).
29. P. James, J. Halladay, E. A. Craig, *Genetics* **144**, 1425-1436 (1996).

30. M. Fromont-Racine, J. Rain, P. Legrain, *Nat Genet* **16**, 277-282 (1997).
31. R. S. Sikorski, J. D. Boeke, *Methods Enzymol.* **194**, 302-318 (1991).
32. S. Enomoto, et al., *Genes & Dev* **11**, 358-370 (1997).
33. P. D. Kaufman, R. Kobayashi, B. Stillman, *Genes & Dev* **11**, 345-357 (1997).
34. L. Sussel, D. Vannier, D. Shore, *Genetics* **141**, 873-888 (1995).
35. D. Vannier, D. Balderes, D. Shore, *Genetics* **144**, 1343-1353 (1996).
36. T. Triolo, R. Sternglantz, *Nature* **381**, 251-253 (1996).
37. F. Palladino, et al., *Cell* **75**, 543-555 (1993).
38. D. Shore, *Trends Genet.* **10**, 408-412 (1994).
39. K. Freeman, M. Gwadz, D. Shore, *Genetics* **141**, 1253-1262 (1995).
40. C. F. Hardy, L. Sussel, L. Sussel, D. Shore, *Genes & Dev* **6**, 801-814 (1992).
41. J. L. Johnson, T. Beito, C. Krco, D. Toft, *Mol. Cell. Biol.* **14**, 1956-1963 (1994).
42. N. Grandin, S. I. Reed, M. Charbonneau, *Genes & Dev* **11**, 512-527 (1997).

FIGURE LEGENDS

Figure 1. The Ku heterodimer, telomerase and the Cdc13 single strand telomere binding protein are each required for full telomere function.

A. Growth of haploid *hdf1-Δ* and *yku80-Δ* mutant strains in the presence or absence of an *est2-Δ* mutation, using equivalent number of cells taken directly from colonies off the dissection plate of freshly dissected diploid strains (after ~25 generations of growth). *yku80-Δ* and *hdf1-Δ* combinations with *est1-Δ*, *tlc1-Δ* and *cdc13-2^{est}* mutations were also tested, with results identical to those shown for *est2-Δ* (data not shown).

B. Growth of haploid *hdf1-Δ* in the presence or absence of the *cdc13-1^{ts}* allele; phenotypes were assessed after ~25 generations of growth of freshly dissected diploid strains. *Yku80-Δ cdc13-1^{ts}* double mutants display a similar phenotype (data not shown).

Figure 2. *RAD50* and *MRE11* are in the telomerase epistasis group.

A. Comparison of telomere length after increasing population doublings, examined after ~25 generations (1x) and ~45 generations (3x). Genomic DNA from cells was digested with *XhoI* and the southern was probed with labelled polyGT/CA to detect telomeric sequences. The broad lower band on the gel represents ~ 2/3 of the telomeres in this strain, whereas the four bands ranging from 1.8 kb to ~4.0 kb correspond to individual telomeres.

B. *mre11-Δ* shows a synthetic phenotype in combination with either *yku80* (top panel, incubated at 23°C), *hdf1-Δ* (data not shown) or with *cdc13-1^{ts}* (incubated at 23°, 26° and 28°C).

Figure 3. The Ku heterodimer, but not Cdc13p or telomerase, is required for silencing of telomere-located genes.

A. Serial 10-fold dilutions of cells from freshly grown wild-type, *cdc13-2^{est}*, *rad50-Δ*, *mre11-Δ*, *hdf1-Δ*, and *sir3-Δ* strains were plated on complete media in order to monitor total cell viability, on media lacking uracil to assess the extent of derepression of *URA3* transcription, and on media containing 5-fluoro-orotic acid (5-FOA) to determine the proportion of cells able to repress *URA3* transcription. Plates were incubated at 30°C for 3 days.

B. Serial 10-fold dilutions of freshly grown wild-type and freshly generated *hdf1-Δ* cells were plated on YPD, -uracil, and 5-FOA and incubated at either 23°C (5 days) or 36°C (2.5 days).

Figure 4. Temperature lethality of *yku80-Δ* or *hdf1-Δ* (data not shown) is suppressed by increased expression of *EST1*, *EST2* or *TLC1*. Wild-type or *yku80-Δ* strains were transformed with: vector alone (pVL399), pVL784 (2μ *pADH-EST1*), pVL999 (2μ *pADH-EST2*), pVL799 (2μ *pADH-TLC1*), pVL411 (2μ *CDC13*, with the native *CDC13* promoter). Cells were grown in selective media and examined at 23°C and 36°C, after sufficient growth to allow manifestation of the Ku-associated temperature sensitive phenotype.

Figure 5. Over-expression of *CDC13* is toxic to either wild-type or *rad9-Δ* cells. Growth of wild-type or *rad9-Δ* cells containing *CDC13* under expression from the *GAL1* promoter or vector alone was assessed by plating 10-fold serial dilutions of cells on media that either maintains repression of the *GAL* promoter (glu = glucose) or induces expression (gal = galactose).

Figure 6. Characterization of *CDC13* two hybrid baits. The various *CDC13* constructs are illustrated, with ovals representing the amino-terminal GAL4-DNA binding domain and HA-tag fusions. (+) indicates the ability of a given construct to either be detected on a western blot, or complement the inviability of *cdc13-Δ*, temperature sensitivity of *cdc13-1^{ts}*, or senescence of *cdc13-2^{est}* mutant strains.

Figure 7. The Stn1p C-terminal region is necessary for Cdc13p interaction. Representative Stn1p-fusion constructs were tested for two-hybrid interaction with pVL705 (encoding Cdc13p with a deletion of its DNA binding domain). The relative strength of the interaction is derived from comparison of the transcriptional activation of the β-galactosidase reporter.

Figure 8. The elongated telomere phenotype of *stn1-13* mutants is dependent upon *yKU80*. Genomic DNA from freshly dissected wild-type, *yku80-Δ*, *stn1-13 yku80-Δ* and *stn1-13* strains was digested with XhoI and run on a 0.8% agarose gel. Loading order: wild-type (lanes 1 and 8), *yku80-Δ* (lanes 2 and 3), *stn1-13 yku80-Δ* (lanes 4 and 5), *stn1-13* (lanes 6 and 7). The southern blot was probed with polyGT /CA to identify telomeric DNA fragments. *stn1-13* telomeres appear to run as heterogeneous, elongated smear.

Table 1. Est2p two-hybrid interacting proteins.

Gene/ORF:	# Isolates:	Comments:
<i>CAC1/RLF2</i>	1	Chromatin assembly complex component/ Rap1 localization factor.
<i>SDS3</i>	1	Extragenic suppressor of defective (rap1-12) silencing.
<i>YLA1</i>	1	Homolog of human La auto-antigen, binds RNA.
<i>TY1</i>	5	TyA (441 aa) encodes "gag" protein of Ty retrotransposon.
<i>JNM1</i>	2	Required for proper nuclear migration during mitosis.
<i>BUB1</i>	1	Serine/threonine protein kinase required for spindle checkpoint.
<i>NIP100</i>	1	Nuclear import protein.
<i>NUP85</i>	1	Nuclear pore complex protein.
<i>YGR280c</i>	4	Lysine and asparagine rich sequence. 31.3 kD, pI=10.
<i>YPR144c</i>	3	Weak similarity to RNA pol β subunit, CCAAT transcription factor.
<i>YIR025w</i>	2	42.8 kD. pI=4.6.
<i>YLR231c</i>	2	ORF neighboring EST1.
<i>YHL046c</i>	2	Sequence similarity to members of Srp1p/Tip1p family.
<i>YDR026c</i>	1	Similarity to REB1; similarity to cmyb DNA bind domain repeat 2.
<i>YLR387c</i>	1	Contains C2H2-type Zn finger domain. 49.7 kD, pI=7.76.
<i>YIL112w</i>	1	123.6 kD. Contains ankyrin repeats.
<i>YPR143w</i>	1	28.2 kD, pI=5.6.
<i>YKL014c</i>	1	203.3 kD, pI=7.2
<i>YLR287c</i>	1	40.9 kD, pI=4.95.
<i>YNL091w</i>	1	141.5 kD, pI=5.26. Similar to protein functioning in golgi.

Prey Plasmid:	Bait Plasmid:		
	<i>CDC13</i>	<i>EST2</i>	<i>EST1</i>
<i>EST1</i>	-	-	-
<i>EST3</i>	-	-	-
<i>CDC13</i>	+-	-	-
<i>RAP1</i> aa 653-end	-	-	-
<i>SIR4</i> aa 771-end	-	-	-
<i>STN1</i>	+	-	-
<i>YKL117w</i>	+	+	-
<i>SDS3</i>	-	+	+
<i>YKU80</i>	-	-	-

Table 2. Directed Two-Hybrid. Specific combinations of genes with telomere related functions were tested for interaction in pJ69-4A. The (+) designations indicate that all three reporter genes were activated in the presence of the indicated bait and prey fusion constructs. The (+-) designation indicates a very weak activation of the three reporter genes.

Figure 1A.

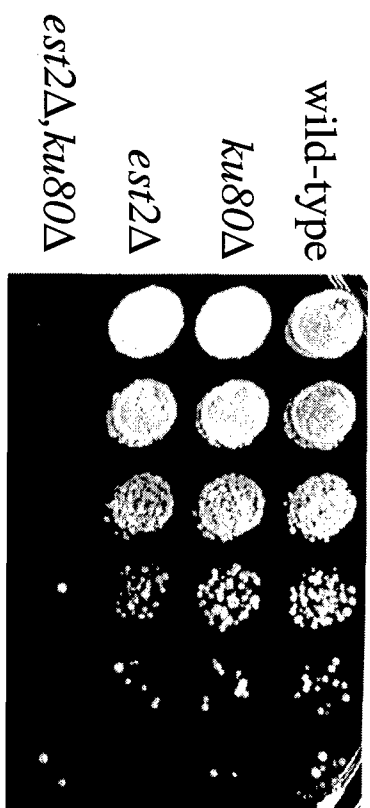
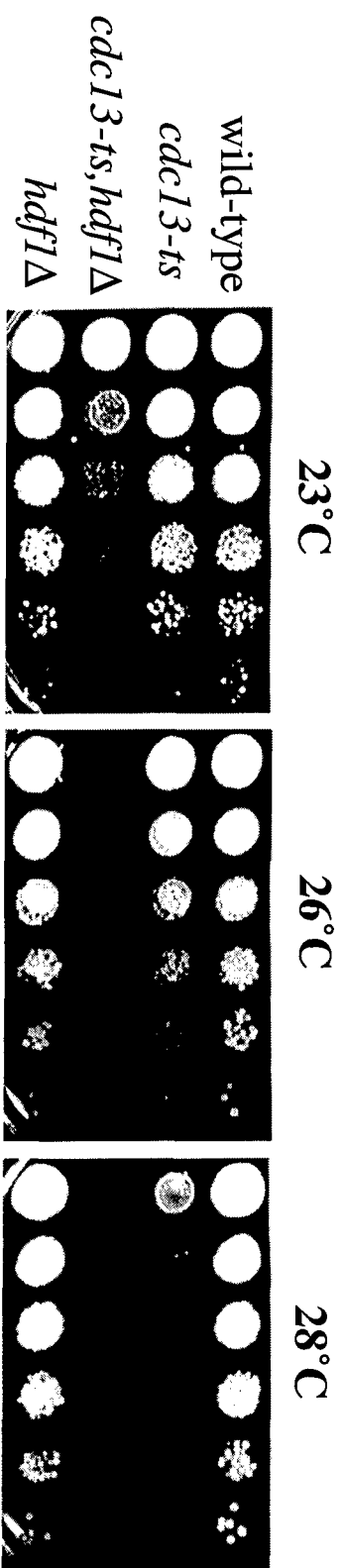


Figure 1B.



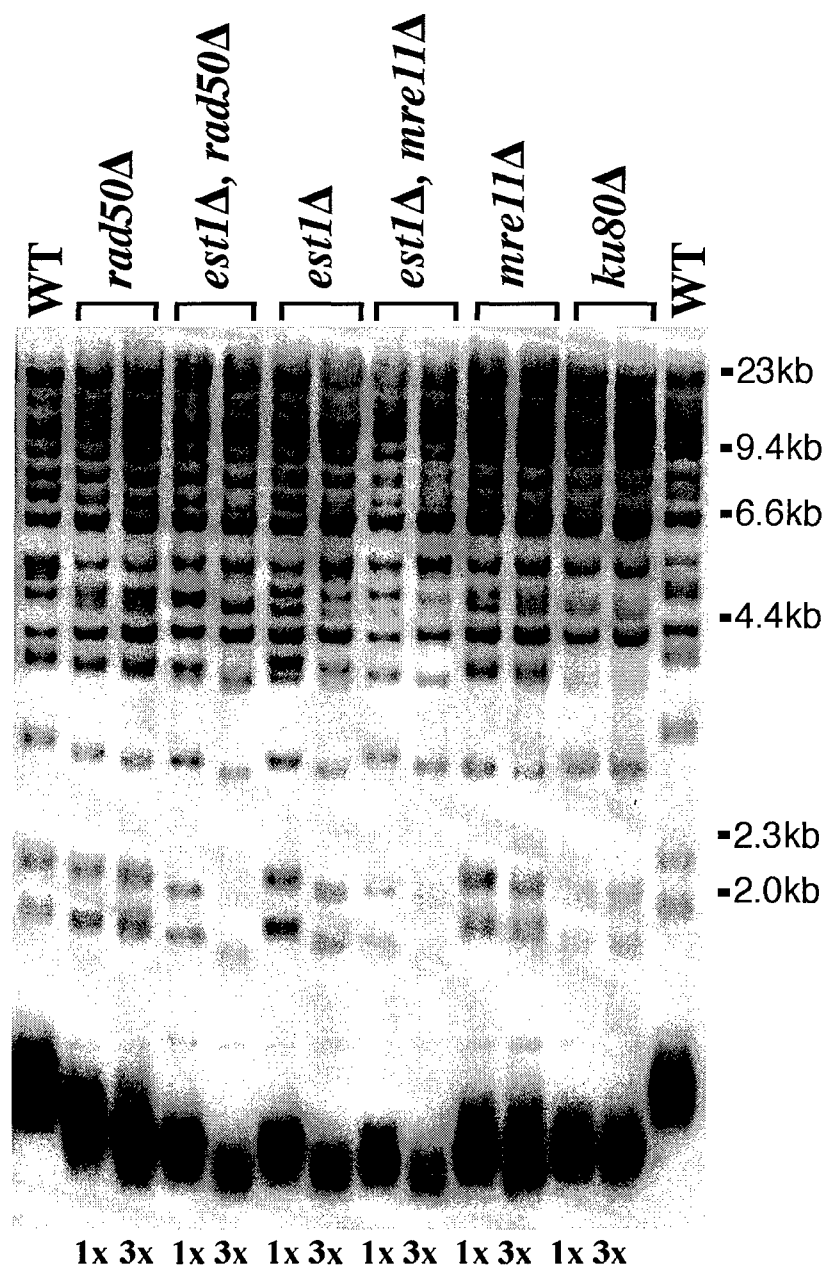


Figure 2A.

Figure 2B.

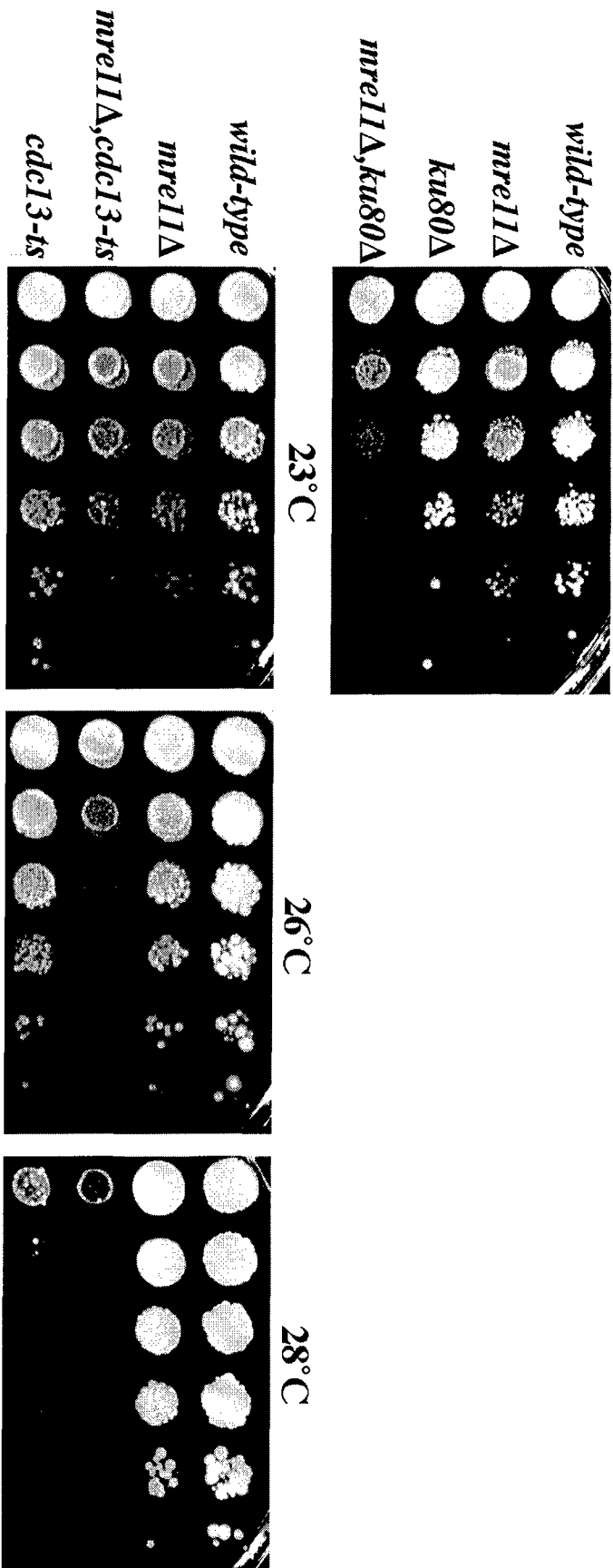


Figure 3A.

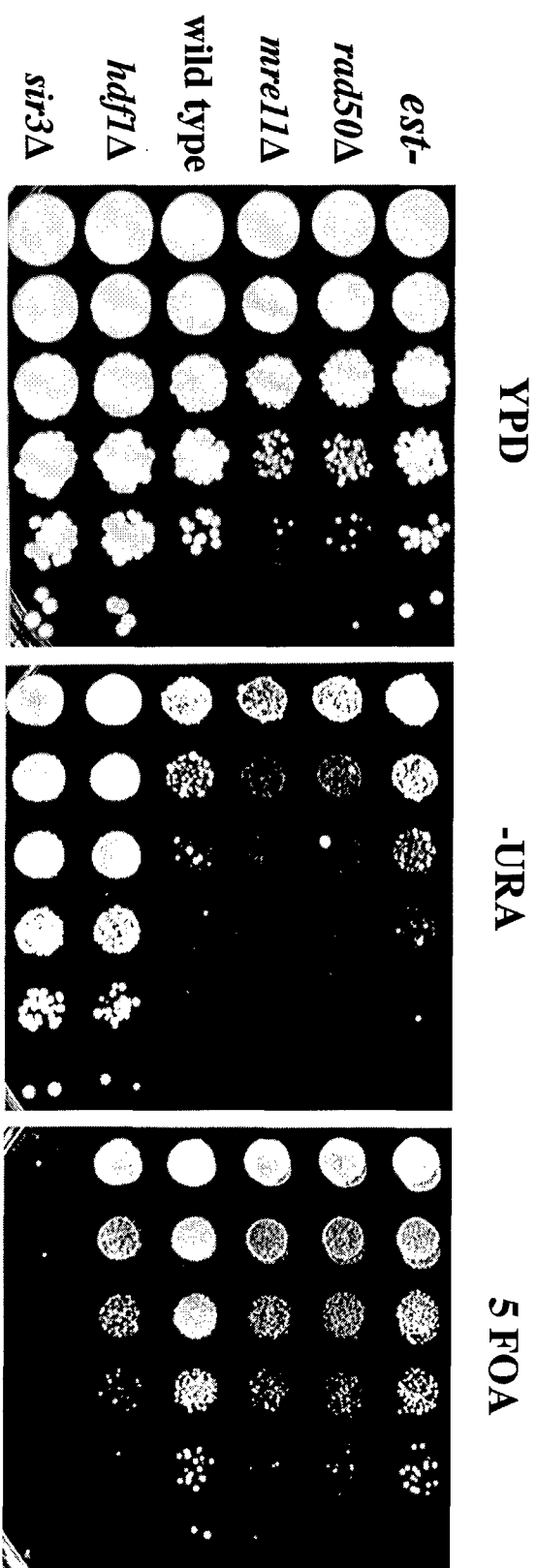
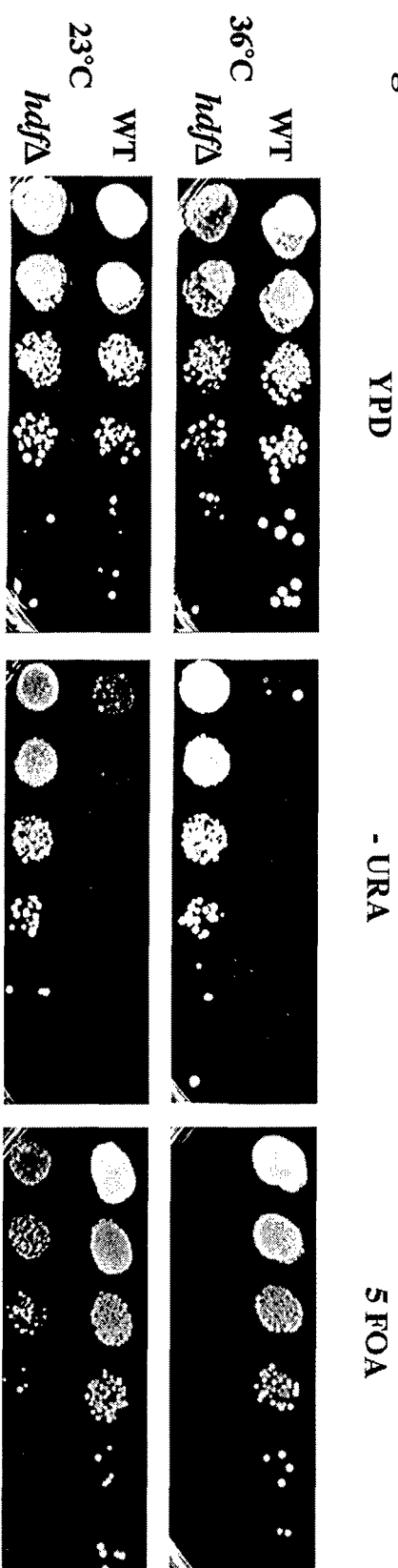


Figure 3B.



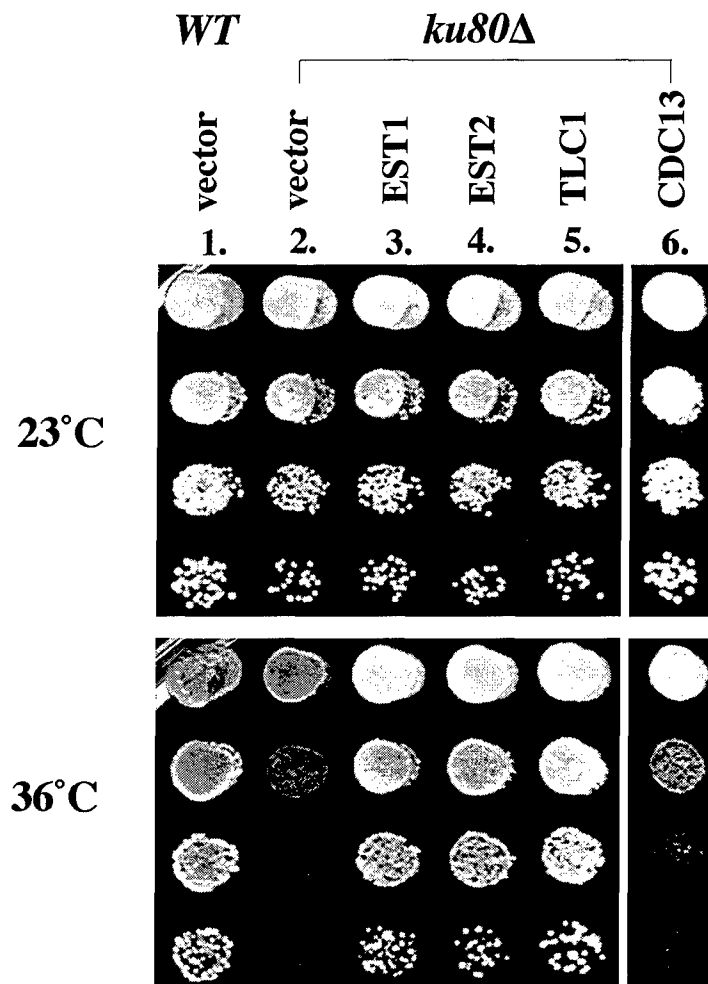


Figure 4.

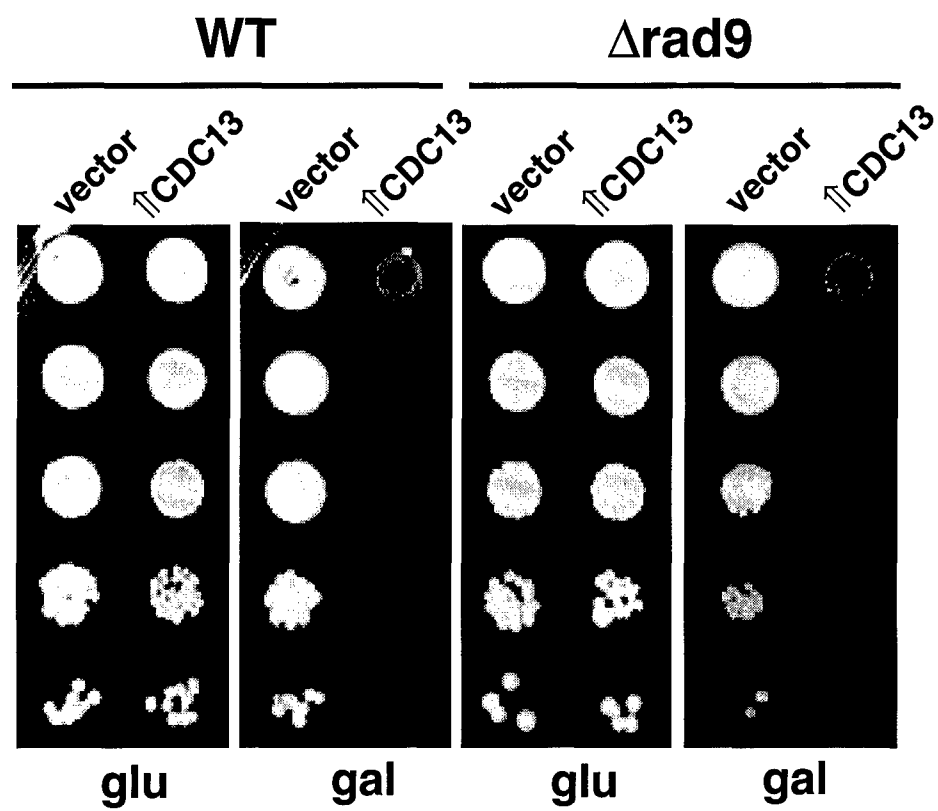
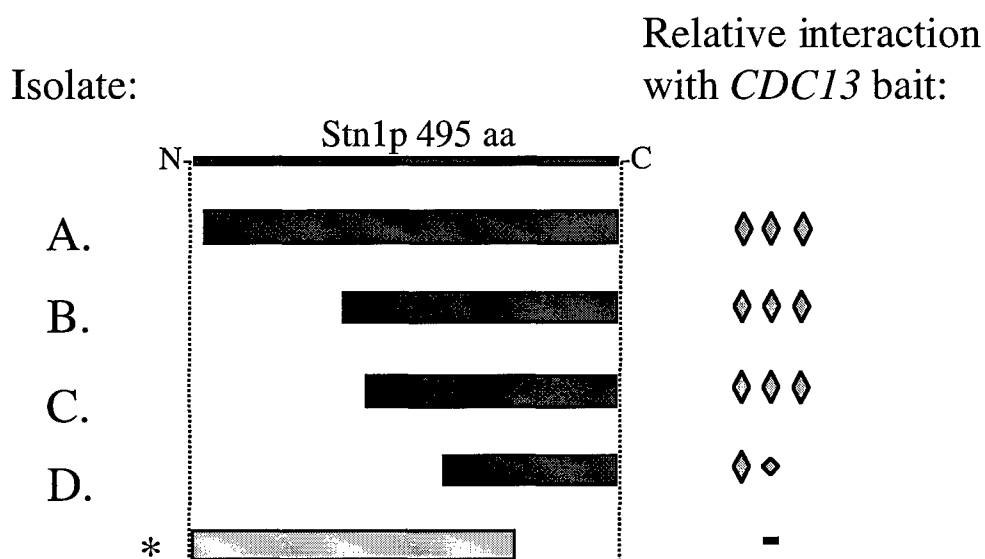


Figure 5. Over-expression of *CDC13* is lethal.

Figure 6. Toxicity and function of Cdc13p Bait Constructs.

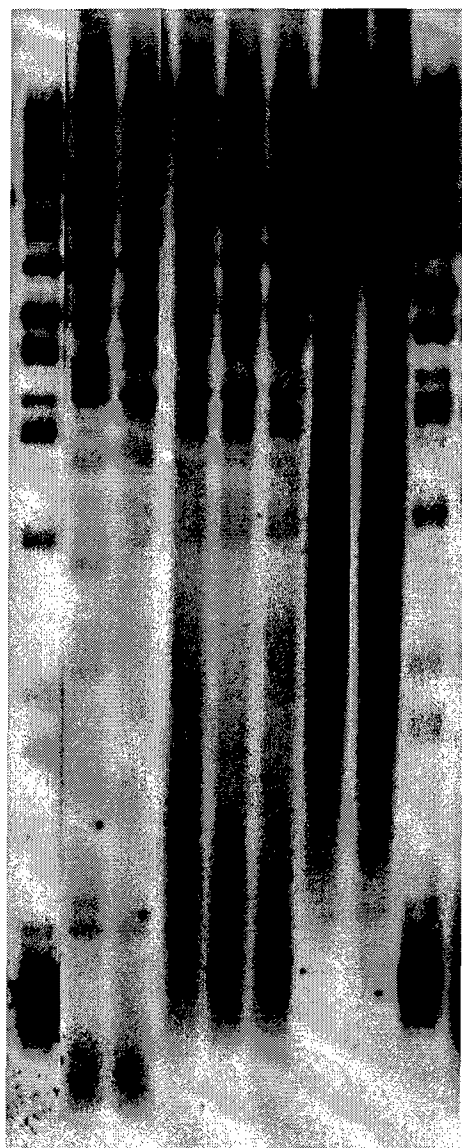
HA- DNA binding domain	TOXIC?	PROTEIN?	COMPLEMENT cdc13 ^{ts} ?	cdc13 ^{ts} ?
	+			
est 	-	-	+	-
ts 	-	+	+	+
	-	-	-	+
	+			
	-	-	-	-
	+			
	+			
	-	+	-	-
☆ 	-	+	-	+

The *CDC13* DNA binding domain appears to be necessary, but not sufficient, for overexpression lethality.



**not isolated through 2-hybrid screen

Figure 7. The Stn1p C-terminal region is necessary for Cdc13p interaction. Representative Stn1p-fusion constructs were tested for two-hybrid interaction with pVL705 (encoding Cdc13p with a deletion of its DNA binding domain). The relative strength of the interaction is derived from comparison of the transcriptional activation of the β -galactosidase reporter.



1. 2. 3. 4. 5. 6. 7. 8. 9.

Figure 8. The *stn1-13* long telomere phenotype is dependent on *yKU80*.

Telomere maintenance is dependent on activities required for end repair of double-strand breaks

Constance I. Nugent*, Giovanni Bosco[†], Lyle O. Ross*, Sara K. Evans[‡], Andrew P. Salinger*, J. Kent Moore[†], James E. Haber[†] and Victoria Lundblad*[‡]

Telomeres are functionally distinct from ends generated by chromosome breakage, in that telomeres, unlike double-strand breaks, are insulated from recombination with other chromosomal termini [1]. We report that the Ku heterodimer and the Rad50/Mre11/Xrs2 complex, both of which are required for repair of double-strand breaks [2–5], have separate roles in normal telomere maintenance in yeast. Using epistasis analysis, we show that the Ku end-binding complex defined a third telomere-associated activity, required in parallel with telomerase [6] and Cdc13, a protein binding the single-strand portion of telomere DNA [7,8]. Furthermore, loss of Ku function altered the expression of telomere-located genes, indicative of a disruption of telomeric chromatin. These data suggest that the Ku complex and the Cdc13 protein function as terminus-binding factors, contributing distinct roles in chromosome end protection. In contrast, *MRE11* and *RAD50* were required for the telomerase-mediated pathway, rather than for telomeric end protection; we propose that this complex functions to prepare DNA ends for telomerase to replicate. These results suggest that as a part of normal telomere maintenance, telomeres are identified as double-strand breaks, with additional mechanisms required to prevent telomere recombination. Ku, Cdc13 and telomerase define three epistasis groups required in parallel for telomere maintenance.

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Results and discussion

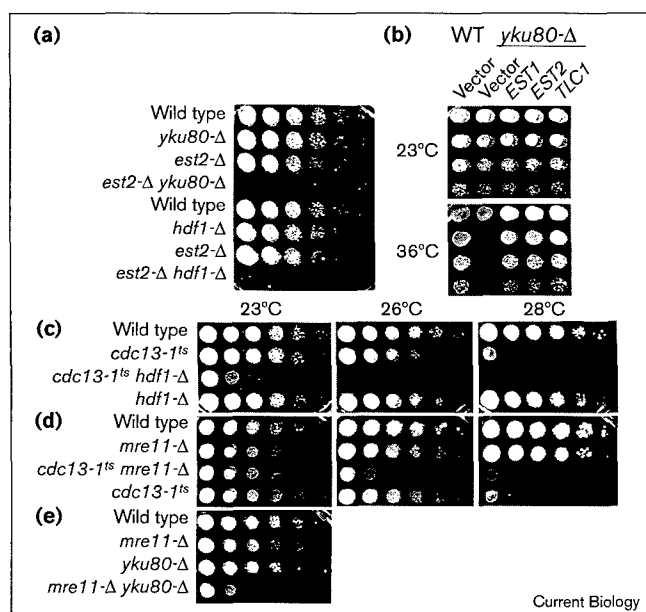
In *Saccharomyces cerevisiae*, telomere replication depends upon the telomerase RNA gene *TLC1* [9], the catalytic subunit *EST2* [10,11], and three other genes, *EST1*, *EST3* and *EST4/CDC13* (reviewed in [6]). Mutations in all five

genes result in senescence, whereby the telomeric repeat tract becomes progressively shorter until telomere function is lost, resulting in chromosome loss and cell death. *CDC13* has also been proposed to separately contribute to telomere end protection in yeast, as the loss of *CDC13* activity that occurs in a *cdc13-1^{ts}* strain results in rapid degradation of the C-rich strand of the telomere and a consequent loss of telomere integrity [12]. Telomere end protection and telomere replication appear to be functionally distinct activities; thus, a telomerase-defective *cdc13-1^{ts}* double mutant strain has an exaggerated growth defect relative to either single mutant strain [7].

We designed two complementary genetic screens to identify additional genes required for telomere replication or integrity. Screen A probed for mutations that lowered the maximum permissive temperature of a *cdc13-1^{ts}* strain, and screen B identified mutations that enhanced the senescence phenotype of an *est1-Δ* mutant strain. As expected, one mutation in *EST1* and two mutations in *EST2* were isolated in screen A that, in combination with the *cdc13-1^{ts}* allele, resulted in enhanced temperature sensitivity and exaggerated senescence. Surprisingly, both screens revealed mutations in genes required for repair of DNA double-strand breaks. Screen A yielded two mutations each in *YKU80* and *RAD50*, and screen B identified two mutations in *YKU80*. Both the Ku heterodimer (encoded by the *YKU80* and *HDF1* genes) and the multiprotein Rad50/Mre11/Xrs2 complex have been shown to be critical for non-homologous DNA double-strand break end-joining repair (NHEJ) [2–5]. Ku binds in a sequence-independent manner with high affinity to the ends of duplex DNA as well as to nicks in double-stranded DNA [3,13]. The precise biochemical function of the Rad50/Mre11/Xrs2 complex in NHEJ is less well understood, with evidence in support of an enzymatic role as a 5' to 3' exonuclease [14,15], or alternatively a more structural role [16].

We then performed directed epistasis tests by examining the phenotypes of strains carrying various mutant combinations, which demonstrated that telomerase, the Ku complex and Cdc13p each contribute distinct roles at the telomere. Any combination of Ku[−] null mutations with deletions of *EST1*, *EST2* or *TLC1* resulted in an exacerbated phenotype, as the double mutant strains all exhibited accelerated inviability (Figure 1a and data not shown). Haploid *hdf1-Δ est2-Δ* and *yku80-Δ est2-Δ* double mutant spores generated from heterozygous diploids gave rise to colonies that consisted of mostly inviable cells, so that

Figure 1



The Ku heterodimer, telomerase and Cdc13 protein are each required for full telomere function. (a) Growth of isogenic haploid *hdf1-Δ* and *yku80-Δ* mutant strains in the presence or absence of an *est2-Δ* mutation, using equivalent numbers of cells taken directly from colonies off the dissection plate of freshly dissected isogenic diploid strains (after ~25 generations of growth). Combinations of *yku80-Δ* and *hdf1-Δ* with *est1-Δ*, *tlc1-Δ* and *cdc13-2^{est}* mutations were also tested, with results identical to those shown for *est2-Δ* (data not shown). (b) Temperature lethality of *yku80-Δ* (or *hdf1-Δ*; data not shown) is suppressed by increased expression of *EST1*, *EST2* or *TLC1*. Wild-type (WT) or *yku80-Δ* strains were transformed with: vector alone (pVL399), pVL784 (2μ *LEU2* pADH-*EST1*), pVL999 (2μ *LEU2* pADH-*EST2*), or pVL799 (2μ *LEU2* pADH-*TLC1*). Cells were grown in selective media and examined at 23°C and 36°C after sufficient growth to allow manifestation of the Ku-associated temperature-sensitive phenotype. (c) Growth of the haploid *hdf1-Δ* strain in the presence or absence of the *cdc13-1^{ts}* allele, with phenotypes assessed after ~25 generations of growth of freshly dissected isogenic diploid strains; *yku80-Δ cdc13-1^{ts}* double mutants display a similar phenotype (data not shown). (d) The *mre11-Δ* strain shows a synthetic phenotype in combination with *cdc13-1^{ts}* (incubated at 23°C, 26°C and 28°C). (e) The *mre11-Δ* strain exhibits a synthetic phenotype in combination with *yku80-Δ*.

such colonies could not be further propagated (Figure 1a). In contrast, *est2-Δ* mutant spores initially exhibited growth comparable to that of wild type, followed by a progressive decrease in cell viability (that is, senescence) [17]. The simplest explanation for this synthetic near-lethality is that increased telomere shortening occurs as a consequence of different mutations impacting on two separate telomere length maintenance pathways. Thus, the telomere shortening defect in Ku-deficient cells is not due to loss of telomerase function, but rather to the loss of another activity required to maintain telomere length. Cells devoid of Ku function show a temperature-sensitive growth phenotype with a phenotypic lag, whereby cells proliferate for a limited number of generations at high temperature before

they die [18]. This temperature-sensitive phenotype of *yku80* mutants could be suppressed by increased expression of *EST1*, *EST2* or *TLC1* (Figure 1b). Therefore, both increases and decreases in the levels of three genes specifically required for telomerase function alter the growth phenotype of mutations in *HDF1* and *YKU80*.

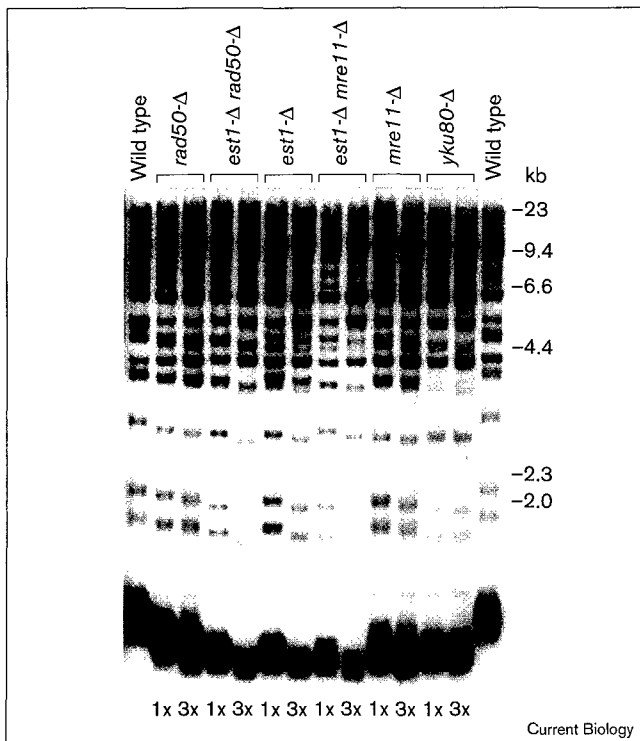
We next examined the effects of combining Ku deletions with the *cdc13-1^{ts}* mutation. As previously observed for *cdc13-1^{ts} tlc1-Δ* double mutants [7], *cdc13-1^{ts} hdf1-Δ* and *cdc13-1^{ts} yku80-Δ* double mutant strains exhibited substantially impaired growth. The maximal permissive temperature of both double mutant strains was reduced relative to the single *cdc13-1^{ts}* strain, and even at permissive temperature, the double mutants had a noticeable growth defect (Figure 1c and data not shown). This synthetic phenotype is not simply the consequence of loss of a Ku-mediated DNA repair pathway required to repair damage generated in the *cdc13-1^{ts}* strain, because a *sir3-Δ* mutation that also eliminates the same DNA end-joining repair pathway [5], did not enhance the defect of a *cdc13-1^{ts}* mutation (data not shown). Therefore, Cdc13p and the Ku proteins, capable of binding single-strand and double-strand DNA substrates, respectively, have separable roles that contribute to telomere integrity.

MRE11 and RAD50 function in the telomerase pathway

The Ku complex and Rad50/Mre11/Xrs2 are both required for efficient DNA end joining and function in a single epistasis group with respect to DNA end joining in yeast [2–5]. Although previous work has shown that deletion of either of these two groups of genes also results in short telomeres [4,19,20], we show here that these genes have strikingly different roles in telomere maintenance. In contrast to *est2-Δ yku80-Δ* and *est2-Δ hdf1-Δ* strains (Figure 1a), mutations in *MRE11* and *RAD50* failed to enhance the telomere replication defect of telomerase mutants. The *mre11-Δ* and *rad50-Δ* strains showed gradual telomere shortening, although the defect was less severe than that observed in *est1-Δ* mutant strains (Figure 2), and *mre11-Δ* and *rad50-Δ* mutations did not confer a senescence phenotype in our strain background (although *rad50-Δ* strains have been reported to exhibit senescence by others [20]). Double mutant strains combining *mre11-Δ* or *rad50-Δ* with either *est1-Δ* or *est2-Δ* mutations did not result in an enhanced loss of either telomere length or cell viability compared to *est1-Δ* or *est2-Δ* single mutant strains (Figure 2 and data not shown). Thus, by these genetic criteria, both Mre11p and Rad50p function in the telomerase-mediated pathway for telomere replication.

Placement of *MRE11* and *RAD50* in the telomerase epistasis group also predicts that mutations in these two genes should behave the same as mutations in *EST1*, *EST2* and *TLC1* with respect to the two other telomere-specific epistasis groups. Consistent with this expectation, *mre11-Δ*

Figure 2



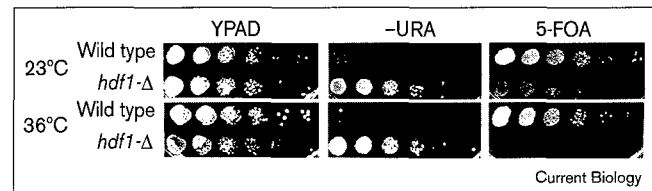
RAD50 and *MRE11* are in the telomerase epistasis group. Comparison of telomere length of isogenic haploid strains after increasing population doublings, examined after ~25 generations (1x) and ~45 generations (3x). The broad 1.2–1.5 kb band represents roughly two-thirds of the telomeres in this strain, and the four bands ranging from 1.8 kb to ~4.0 kb correspond to individual telomeres.

and *rad50-Δ* mutations exhibited enhanced phenotypes with either *cdc13-1^{ts}* mutations or Ku⁻ mutations. Introduction of a *mre11-Δ* or *rad50-Δ* mutation into a *cdc13-1^{ts}* strain reduced the maximum permissive temperature (Figure 1d and data not shown). Similarly, double mutant combinations of *mre11-Δ* or *rad50-Δ* with *hdf1-Δ* or *yku80-Δ* have decreased viability (Figure 1e and data not shown), although the synthetic defect is not as severe as for *est2-Δ yku80-Δ* double mutants (Figure 1a).

Absence of Ku function relieves repression of telomere-located genes

Telomere-localized reporter genes are subject to reversible transcriptional repression [1], referred to as telomeric position effect (TPE). In addition to structural components of telomeric chromatin that are required for TPE, genetic analysis has predicted the existence of a terminus-specific binding factor that is critical for TPE [21]. Three candidates for terminus-specific activities are Cdc13p, the Ku heterodimer, and telomerase itself. We therefore examined the effects of mutations in each of these epistasis groups for effects on TPE. Expression of the *URA3* gene when placed next to the telomere can be monitored via either

Figure 3



The Ku heterodimer is required for silencing of a telomere-located gene. The extent of repression of a telomeric *URA3* reporter gene [9] was measured by plating serial 10-fold dilutions of cells from wild-type and *hdf1-Δ* cells, assessed immediately after dissection of an *HDF1/hdf1-Δ* diploid strain, on complete media (YPAD) to monitor total cell viability, on media lacking uracil (-URA) to assess the extent of derepression of *URA3* transcription, and on media containing 5-FOA to determine the proportion of cells able to repress *URA3* transcription. Plates were incubated at either 23°C (5 days) or 36°C (2.5 days).

growth in the absence of uracil or growth in the presence of a drug inhibitory to Ura⁺ cells (5-fluoro-orotic acid, 5-FOA) [9]. Elimination of telomerase function by deletion of *TLC1* or *EST2* or by mutating the telomerase function of *CDC13* (*cdc13-2^{est}*) [7,17] did not alter the level of repression of *URA3* compared to the complete derepression that occurs in the absence of the Sir3 protein (Supplementary material and data not shown). Similarly, *mre11-Δ* and *rad50-Δ* deletions showed no effects on TPE (Supplementary material), consistent with the placement of these two genes in the telomerase epistasis group. Moreover, *cdc13-1^{ts}*, which disrupts a telomere-binding function distinct from the telomerase defect of the *cdc13-2^{est}* allele [7,12], did not alter TPE at either permissive or semi-permissive temperatures ([8] and data not shown).

In contrast, TPE is substantially altered by the loss of the Ku heterodimer. At temperatures permissive for long-term growth (23°C), elimination of Ku resulted in an intermediate effect on *URA3* expression (Figure 3), suggesting that the repressed state is not adequately maintained. An even more severe defect was observed when TPE was examined in an *hdf1-Δ* strain immediately after transfer to 36°C, when the strain was still viable: repression of the telomere-located *URA3* reporter gene was now completely abolished (Figure 3), comparable to results observed when an essential component of telomeric chromatin, Sir3p, is deleted. Thus, the Ku complex appears to play a crucial role in maintaining telomeric chromatin structure, consistent with the prediction for a telomere end-binding activity. This role for Ku is partially redundant at low temperatures, however, with some other unidentified activity at the telomere that is itself temperature-labile.

Our results demonstrate that Ku proteins define a discrete telomere-dependent function that is required in parallel with *CDC13* and telomerase. We further propose that the Ku complex and Cdc13p are terminus-specific proteins

that collaborate to protect the end of the chromosome, and that the inability of a telomerase-defective strain to replicate the terminus, when combined with loss of the activity of Cdc13p or Ku, is catastrophic for telomere maintenance. Several observations support the proposal of two activities required for end protection. First, the biochemical properties of both proteins are consistent with an *in vivo* role in terminus binding: Ku has an affinity for duplex DNA ends [13], whereas Cdc13 binds to single-stranded G₁₋₃T DNA [7,8]. Second, mutations in both lead to alterations in telomeric end structure: loss of *CDC13* function results in removal of the C-rich strand of the telomere and consequent lethality [12], and regulation of the S-phase-specific chromosomal end structure is disrupted in cells that lack Ku function [22]. Loss of Ku function also alters the expression of telomeric reporter genes ([4,23]; this work), as predicted for a telomere end-binding protein [21]. This proposed role for the Ku heterodimer at the telomere is also distinct from that of the Sir complex. Although Sir⁻ mutations also derepress TPE and DNA joining [1,5], and Hdf1p interacts with Sir4p [5], *sir* mutants do not enhance the temperature-sensitivity of either *hdf1* or *cdc13-ts* mutations (data not shown).

In contrast, the Rad50/Mre11/Xrs2 complex appears to play a role in mediating replication of telomeres via the telomerase pathway. That they are in the same epistasis group as telomerase suggests that they may function to prepare or present DNA ends to telomerase for further replication. Based on a reduced rate of 5' to 3' exonucleolytic strand processing of DNA ends with *rad50*, *mre11* and *xrs2* mutations [14,15], this complex has been proposed to have exonuclease activity; if so, these proteins may prepare a single-strand substrate that can be acted upon by telomerase, as telomerase cannot extend a duplex blunt end.

These results indicate that gene products previously implicated in repairing double-strand breaks are also directly involved at another terminus, the telomere. However, a critical difference is that telomeres do not normally allow recombination or end-to-end joining with other chromosome ends. Additional telomere-specific factors, such as the Cdc13 protein, may alter the roles of these proteins when present at the telomere. Further characterization of these proteins in both double-strand break repair and telomere function will be necessary to reveal the similarities and differences in how these two different types of DNA ends are processed.

Supplementary material

A figure showing that neither Cdc13p nor telomerase are required for silencing of a telomere-located gene and additional methodological details are published with this article on the internet.

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References

1. Zakian VA: **Structure, function, and replication of *Saccharomyces cerevisiae* telomeres.** *Annu Rev Genet* 1996, **30**:141-172.
2. Moore JK, Haber JE: **Cell cycle and genetic requirements of two pathways of nonhomologous end-joining repair of double-strand breaks in *Saccharomyces cerevisiae*.** *Mol Cell Biol* 1996, **16**:2164-2173.
3. Milne GT, Jin S, Shannon KB, Weaver DT: **Mutations in two Ku homologs define a DNA end-joining repair pathway in *Saccharomyces cerevisiae*.** *Mol Cell Biol* 1996, **16**:4189-4198.
4. Boulton SJ, Jackson SP: **Components of the Ku-dependent non-homologous end-joining pathway are involved in telomeric length maintenance and telomeric silencing.** *EMBO J* 1998, **17**:1819-1828.
5. Tsukamoto Y, Kato J, Ikeda H: **Silencing factors participate in DNA repair and recombination in *Saccharomyces cerevisiae*.** *Nature* 1997, **388**:900-903.
6. Nugent CI, Lundblad V: **The telomerase reverse transcriptase: components and regulation.** *Genes Dev* 1998, **12**:1073-1085.
7. Nugent CI, Hughes TR, Lue NF, Lundblad V: **Cdc13p: a single-strand telomeric DNA-binding protein with a dual role in yeast telomere maintenance.** *Science* 1996, **274**:249-252.
8. Lin JJ, Zakian VA: **The *Saccharomyces* CDC13 protein is a single-strand TG1-3 telomeric DNA-binding protein *in vitro* that affects telomere behavior *in vivo*.** *Proc Natl Acad Sci USA* 1996, **93**:13760-13765.
9. Singer MS, Gottschling DE: **TLC1: template RNA component of *Saccharomyces cerevisiae* telomerase.** *Science* 1994, **266**:404-409.
10. Lingner J, Hughes TR, Shevchenko A, Mann M, Lundblad V, Cech TR: **Reverse transcriptase motifs in the catalytic subunit of telomerase.** *Science* 1997, **276**:561-567.
11. Counter CM, Meyerson M, Eaton EN, Weinberg RA: **The catalytic subunit of yeast telomerase.** *Proc Natl Acad Sci USA* 1997, **94**:9202-9207.
12. Garvik B, Carson M, Hartwell L: **Single-stranded DNA arising at telomeres in *cdc13* mutants may constitute a specific signal for the RAD9 checkpoint.** *Mol Cell Biol* 1995, **15**:6128-6138.
13. Lieber MR, Grawunder U, Wu X, Yaneva M: **Tying loose ends: roles of Ku and DNA-dependent protein kinase in the repair of double-strand breaks.** *Curr Opin Genet Dev* 1997, **7**:99-104.
14. Tsubouchi H, Ogawa H: **A novel *mre11* mutation impairs processing of double-strand breaks of DNA during both mitosis and meiosis.** *Mol Cell Biol* 1998, **18**:260-268.
15. Ivanov EL, Sugawara N, White CI, Fabre F, Haber JE: **Mutations in *XRS2* and *RAD50* delay but do not prevent mating-type switching in *Saccharomyces cerevisiae*.** *Mol Cell Biol* 1994, **14**:3414-3425.
16. Kleckner N: **Meiosis: how could it work?** *Proc Natl Acad Sci USA* 1996, **93**:8167-8174.
17. Lendvay TS, Morris DK, Sah J, Balasubramanian B, Lundblad V: **Senescence mutants of *Saccharomyces cerevisiae* with a defect in telomere replication identify three additional *EST* genes.** *Genetics* 1996, **144**:1399-1412.
18. Barnes G, Rio D: **DNA double-strand-break sensitivity, DNA replication, and cell cycle arrest phenotypes of Ku-deficient *Saccharomyces cerevisiae*.** *Proc Natl Acad Sci USA* 1997, **94**:867-872.
19. Porter SE, Greenwell PW, Ritchie KB, Petes TD: **The DNA-binding protein Hdf1p (a putative Ku homologue) is required for maintaining normal telomere length in *Saccharomyces cerevisiae*.** *Nucleic Acids Res* 1996, **24**:582-585.
20. Kironmai KM, Muniyappa K: **Alteration of telomeric sequences and senescence caused by mutations in *RAD50* of *Saccharomyces cerevisiae*.** *Genes Cells* 1997, **2**:443-455.
21. Wiley EA, Zakian VA: **Extra telomeres, but not internal tracts of telomeric DNA, reduce transcriptional repression at *Saccharomyces* telomeres.** *Genetics* 1995, **139**:67-79.
22. Gravel S, Larrivee M, Labrecque P, Wellinger RJ: **The yeast Ku complex as a regulator of the chromosomal DNA end-structure.** *Science* 1998, in press.
23. Laroche T, Martin SG, Gotta M, Gorham HC, Pryde FE, Louis EJ, Gasser SM: **Mutation of yeast Ku genes disrupts the subnuclear organization of telomeres.** *Curr Biol* 1998, **8**:653-656.

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