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Telomeres are the protective ends of chromosomes that are necessary for maintaining the viability of a cell and the integrity of its genome. One essential function of telomeres is to protect the chromosome end from being degraded by nucleases that are normally present in any cell. Unprotected chromosome ends could fuse to each other leading to loss of genetic material, genomic instability, and subsequent carcinogenesis. The focus of my research is to understand the mechanism of chromosome end protection in the well-studied model organism, *S. cerevisiae*, and then extend the knowledge we gain in yeast to humans by identifying human homologues of the essential proteins involved in end protection. Using molecular and phylogenetic approaches, I have successfully identified yeast homologues of three proteins known to be involved in end protection – Cdc13, Stn1 and Ten1. With these homologues in hand, I have initiated searches for human homologues of Stn1 and Ten1. Using a combination of bioinformatics and yeast genetics, I have potentially identified a site of interaction between Cdc13 and Stn1 that is essential for mediating end protection. These findings have further elucidated the mechanism of end protection and have allowed us to start looking for similar proteins and parallel mechanisms in humans.

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## Table of Contents

## Introduction

Telomeres are the protective ends of chromosomes that are necessary for maintaining the viability of a cell and the integrity of its genome. Telomeres shorten with every cell division and eventually diminish leading to cell death. In order to repair these telomere ends and avoid cell death, cells can use telomerase to elongate these protective ends. Normal breast tissue that does not express telomerase and therefore has a finite lifespan while breast cancer cells with aberrant telomerase expression ignore this pre-programmed death and can proliferate continuously. Telomeres must also protect the chromosome end from being degraded by nucleases that are normally present in any cell. Unprotected chromosome ends could fuse to each other leading to loss of genetic material and genomic instability. Genomic instability, such as that caused by chromosome fusions, contributes substantially to the stepwise progression of mammary carcinogenesis.

In the budding yeast, *S. cerevisiae,* Cdc13 plays a critical role in both telomere length regulation and, the focus of my research, telomere end protection[1]. Work from the Lundblad and Charbonneau labs has shown that Cdc13 and the end protection complex, including Stn1 and Ten1, are critical regulators of telomere function[2-4] Given the essential role of Cdc13 and subunits of the end protection complex in yeast, these proteins are likely to have functional homologues in humans which, when identified, can aid in the development of therapeutic agents for the treatment of breast cancer.

## Body

In contrast to the situation in mammalian cells, a number of genes have been identified that are required for telomerase function in *S. cerevisiae*, including Cdc13, Stn1 and Ten1[2-4]. Mutations in any of these factors results in extensive degradation of the chromosome end and eventual cell death. The goal of my research is to first take advantage of the rapid and efficient methods established for *S. cerevisiae* in order to further understand the mechanism of end protection in eukaryotes and then extend the knowledge we gain in yeast to humans by identifying human homologues of these essential proteins.

While the main goal of my research remains unchanged, recent advances in the telomere biology field have steered a subset of the specific tasks – particularly those related to the formation of t-loops - in another direction. T-loops are formed at telomeres when the 3' single strand telomeric overhang invades the duplex region of the telomere – an architectural conformation that is proposed to serve in end protection[5]. T-loops had previously only been identified in trypanosomes, mice and humans [5, 6]. As such, it was of interest to know whether this mechanism of end protection was conserved in yeast. Recently, Tomaska and Griffith have used electron microscopy to identify the formation of such lariat structures in both budding and fission yeast (unpublished). Their results further implicate Taz1, a pombe telomere binding protein related to the human TRF proteins, as being a key mediator in the formation of these structures. The results presented by the Griffith lab as well as some exciting data I've obtained in the past few months have made it logical to re-prioritize my research and revise my statement of work which is appended to this report and has been submitted for approval. Of the six tasks proposed, one is completed and two should be completed in the near future.

### *Task 1.* Identification of yeast homologues of the end protection complex.

Through our phylogenetic studies, I have successfully identified the full length sequences of homologues of the essential protein, Stn1 in the following budding yeast species: *S. castellii, , S. kluyveri,* and *S. kudriazevii* (Fig. 1, 2a). In collaboration with Mike McEachern, we have also obtained a homologue in the more evolutionary diverged *K. lactis.* I have also identified fragments of Stn1 homologues in *S. bayanus,* and *S. mikatae.* Similarly, I have successfully identified homologues of

Ten1 in the following budding yeast species: *S. bayanus, S. paradoxus, S. mikatae, S. castellii,* and *S. kudriazevii* (Fig. 1, 2b).

In addition to identifying Stn1 and Ten1 homologues, I have used similar methods to identify Cdc13 homologues in the following species: *S. paradoxus, S. bayanus, S. mikatae, S. castellii, S. kudriazevii, S. kluyveri,* and *K. thermotolerans* (Fig. 1, 2c). These are bonafide homologues as one of the most evolutionarily divergent homologues, *S. kluyveri,* complements the viability of a *cdc13-Δ S. cerevisiae* strain and is able to stably maintain telomeres be it at a longer length (data not shown). I am currently in the process of using similar methods to identify a *K. lactis* homologue of Cdc13.

**Figure 1. Yeast phylogenetic tree.** Phylogenetic tree based on rDNA sequences of the depicted budding yeast species.

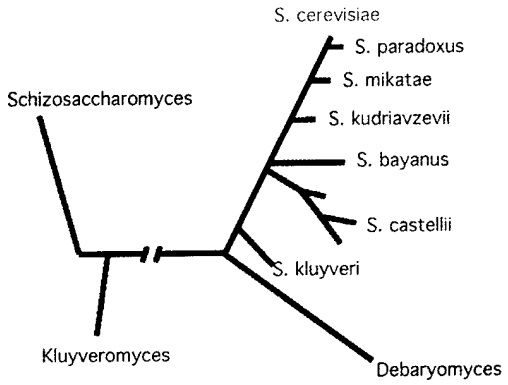


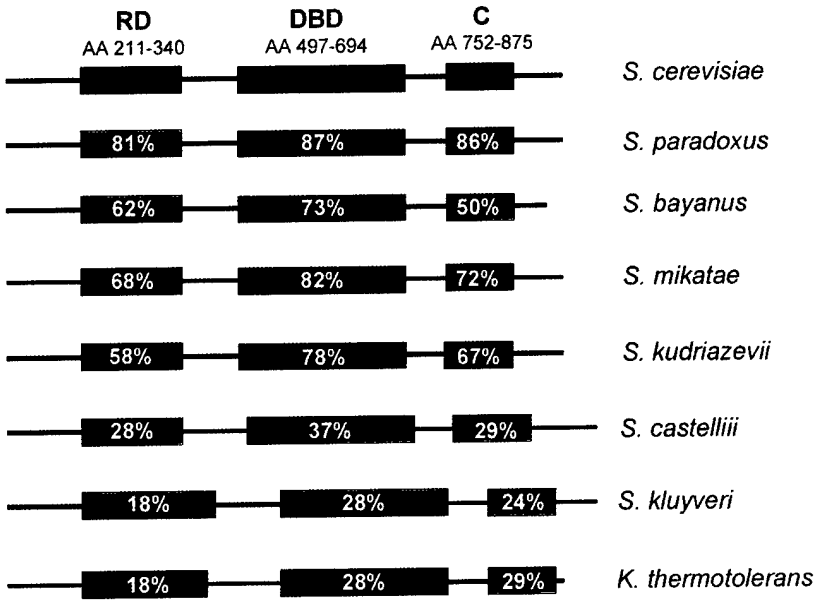

**Figure 2a. Stn1 homologue % identity.** Stn1 homologues were identified in four budding yeast species. Table below shows both % identity and % similarity between the organism listed and *S. cerevisiae* over the entire length of the protein.

| Organism | % identity | % similarity |
|---|---|---|
| S. kudriazevii | 66 | 33 |
| S. castellii | 30 | 21 |
| S. kluyveri | 26 | 19 |
| K. lactis | 16 | 17 |

**Figure 2b. Ten1 homologue % identity.** Ten1 homologues were identified in five budding yeast species. Table below shows both % identity and % similarity between the organism listed and *S. cerevisiae* over the entire length of the protein.

| Organism | % identity | % similarity |
|---|---|---|
| S. paradoxus | 80 | 5 |
| S. bayanus | 66 | 10 |
| S. mikatae | 69 | 10 |
| S. kudriazevii | 66 | 9 |
| S. castellii | 29 | 17 |

**Figure 2c. Cdc13 homologues % identity.** Cdc13 homologues were identified in seven budding yeast species. The schematic below shows % identity between the organism listed and *S. cerevisiae* within the domains depicted below. RD: recruitment domain, ~130 amino acid domain of Cdc13 that our lab proposes recruits telomerase to the telomere via an interaction with the Est1 subunit; DBD: DNA binding domain, 200 amino acid domain of Cdc13 that our lab and the Wuttke lab has demonstrated is necessary and sufficient for binding telomeric DNA; and C: a domain of unidentified function that is deleted in the *cdc13-5* allele[7].




***Task 2.* Identify human homologues of the end protection complex.**

Pot1, a single strand telomere binding protein, was originally identified in fission yeast and humans based on its homology to the α subunit of the ciliate telomere binding protein (TEBPα)[8]. At the time of submission of this grant, it was unclear as to whether Pot1 was the functional human homologue of Cdc13. However, the elucidation of the structure of the DNA binding domain of Cdc13 by the Wuttke lab in collaboration with our lab[9] has suggested that while there is no obvious sequence similarity between the two proteins, there is strong structural homology. With increasing data supporting Pot1 as a human homologue of Cdc13, it is of even greater interest to identify the human homologues of other components of the end protection complex, specifically Stn1 and Ten1.

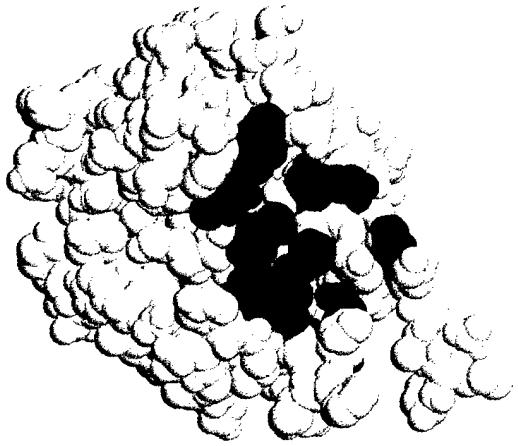
While there are numerous methods to align homologous sequences, Hidden Markov Model (HMM)-based multiple sequence alignments use statistics to consider all possible combinations of matches, mismatches and gaps to generate an alignment of a set of sequences[10]. I used Hidden Markov Modeling to align the sequences of the various budding yeast Ten1 homologues and then created a sequence profile in order to search for human homologues. Similar studies of Stn1 are being conducted in collaboration with a graduate student in the lab. Using sequence profiles based only on budding yeast sequences, there are no obvious human homologues of either Stn1 or Ten1. However, I am currently using these budding yeast-based profiles to search genome databases of other organisms in an attempt to identify additional, non-yeast homologues and eventually "walk" my way toward human homologues of both proteins.

***Task 3.*** **Investigate the mechanism by which Cdc13 regulates telomere end protection.**

## a. Perform an evolutionary trace of identified Cdc13 homologues

An evolutionary trace is a computational method that predicts active sites and functional interfaces in proteins with known structure[11]. The method identifies functionally important residues from sequence conservation patterns and phylogenetic history and maps them onto the protein structure to generate clusters and identify functional interfaces. Using the homologues listed in Task 1 and the recently elucidated structure of the Cdc13 DNA binding domain[9], I collaborated with the Lichtarge lab to carry out an evolutionary trace of Cdc13. The evolutionary trace predicted a functional site on one face of the Cdc13 DNA binding domain that was adjacent to and only partially overlapped with the identified DNA contact site[12, 13] (Fig. 3).

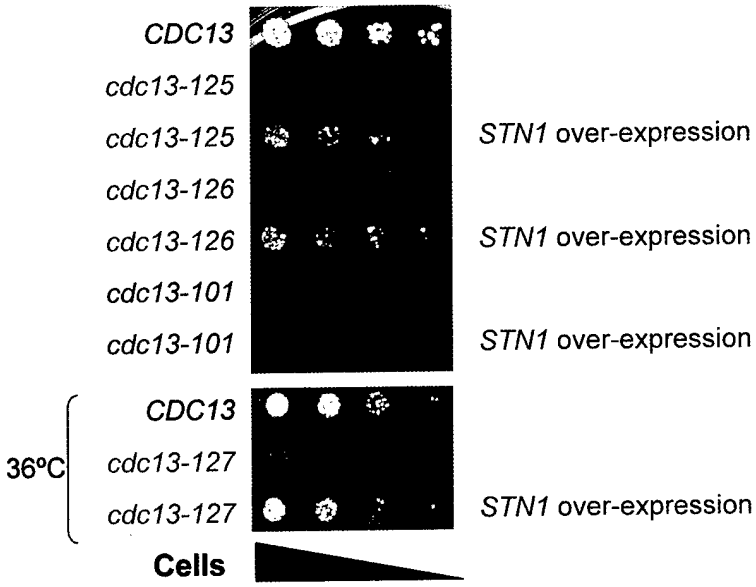
**Figure 3. Structure with predicted functional site.** An evolutionary trace performed on the seven budding yeast Cdc13 homologues and the structure of the Cdc13 DNA binding domain predicted a functional site on one face of the structure. Experiments described in this report support the idea that this functional site composes a site of essential interaction between Cdc13 and Stn1.



## b. Conduct mutational analysis of amino acid residues identified by the Cdc13 evolutionary trace

Directed by the evolutionary trace, I performed mutational analysis on the residues identified by the trace and looked at their *in vivo* phenotype. A subset of the created alleles were lethal (*cdc13-125 and cdc13-126)* and one was temperature sensitive (*cdc13-127*) (Fig. 4). Our lab has previously proposed that end protection is mediated by an essential interaction between Cdc13 and Stn1[2]. It was therefore of interest to test whether these phenotypic cdc13 alleles (collectively referred to as $cdc13^{ET}$) are defective in their interaction with Stn1 by asking what the effects of Stn1 overexpression are in these mutants. Strikingly, overexpression of Stn1 strongly suppressed the lethality of the $cdc13^{ET}$ mutants but not mutants that are lethal due to a defect in general DNA binding (*cdc13-101,* unpublished results and [12]) (Fig. 4). These data suggest that the $cdc13^{ET}$ mutants are deficient in a direct or indirect interaction with Stn1. To more directly test this hypothesis, I am currently looking for alleles of Stn1 that specifically suppress the $cdc13^{ET}$ mutants. Identification of such an allele would suggest that it is a direct interaction between the two proteins that is interrupted in the $cdc13^{ET}$ mutants. Our lab has previously used a similar approach to demonstrate a direct interaction between Cdc13 and a subunit of the telomerase complex, Est1[2].

**Figure 4. Overexpression of Stn1 suppresses the lethal phenotype of a subset of cdc13$^{ET}$ mutants.** A plasmid containing the mutant cdc13 was transformed into a strain lacking its genomic copy of wild type Cdc13. Below is a colony forming ability assay of the cdc13$^{ET}$ mutants (*cdc13-125, cdc13-126,* and *cdc13-127*) and a DNA binding defective mutant (*cdc13-101)* as 5-fold serial dilutions. Row 1: Wild type control; row 2: *cdc13-125* (lethal); row 3: *cdc13-125* viability rescued by overexpression of Stn1; row 4: *cdc13-126* (lethal); row 5: *cdc13-126* viability rescued by overexpression of Stn1; row 6: *cdc13-101* (lethal); row 7: *cdc13-101* lethality not rescued by Stn1 overexpression; row 8: Wild type control at 36°C; row 9: *cdc13-127* (lethal at 36°C); row 10: *cdc13-127* lethality rescued by Stn1 overexpression.




## c. Conduct mutational analysis of highly conserved amino acid residues of Ten1 and Stn1

In order to further our understanding of how these proteins mediate end protection, I wanted to identify key residues of Ten1 that play a role in this regard. Because the structure of both Stn1 and Ten1 have yet to be elucidated, we are not able to perform an evolutionary trace like we did for Cdc13. Instead, I chose to mutate highly conserved residues and analyzed their *in vivo* phenotype. Mutations at two specific residues appear to cause lethal phenotypes I am currently testing to see if these mutants are defective in their interaction with Cdc13 or Stn1.

## Key Research Accomplishments

Identified four budding yeast homologues of Stn1
Identified five budding yeast homologues of Ten1
Identified seven budding yeast homologues of Cdc13
Initiated search for human homologues of Stn1 and Ten1
Performed mutational analysis of Cdc13 residues predicted to form a functional site by the evolutionary trace.
Found three residues that may compose the essential site of interaction between Cdc13 and the end protection complex via Stn1.

## Reportable Outcomes

Presentations:
"Identification of two functional distinct classes of residues within the DNA binding domain of Cdc13" Cold Spring Harbor Laboratory Meeting on Telomeres & Telomerase, Cold Spring Harbor, New York. May 2003. Oral Presentation.

## Conclusions

The research completed under this grant has focused on (1) understanding the mechanisms of chromosome end protection; and (2) identifying human homologues of known components of the end protection complex. More specifically, this research has led to the identification of yeast homologues of all three components of the end protection complex – Cdc13, Stn1 and Ten1 – therefore providing a more sophisticated tool for searching for human homologues of each protein. In addition, I have potentially identified the domain of interaction between Cdc13 and Stn1. This finding is significant as regulation of this interaction will help shed light on how end protection is mediated and leads us to ask whether such a mechanism is conserved in higher eukaryotes. Given the significance of this recent data along with reported results from other labs, I will need to revise my Statement of Work as I will be further investigating the significance and regulation of this interaction.

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## REVISED STATEMENT OF WORK

***Task 1.*** Identify a collection of yeast homologues of the end protection complex. (Months 1-9)

a. Construct *stn1*Δ and *ten1*Δ strains.
b. Transform each of the already constructed budding yeast genomic DNA libraries into *stn1*Δ and *ten1*Δ strains and recover complementing plasmids using standard yeast protocols.
c. Sequence complementing inserts from both screens.

***Task 2.*** Identify human homologues of the end protection complex. (Months 10-12)

a. Align sequences and use conserved regions to search for human homologues of Stn1 and Ten1 in databases.

***Task 3.*** Investigate the mechanism by which Cdc13 regulates telomere end protection (Months 9-18)

a. Perform an evolutionary trace of identified Cdc13 homologues
b. Conduct mutational analysis of amino acid residues identified by the Cdc13 evolutionary trace
c. Conduct mutational analysis of highly conserved amino acid residues of Ten1 and Stn1

***Task 4.*** Determine whether the localization of Cdc13, Stn1 and Ten1 to the telomere is temporally regulated and if phosphorylation affects its telomere localization. (Months 18-24)

a. Perform ChIP assays on Cdc13, Stn1 and Ten1.
b. Use immunoprecipitations and Western blots to determine if the three proteins are phosphorylated in a cell cycle regulated manner.
c. Identify phosphorylated residues by mass spectroscopy.
d. Mutate these phosphorylated sites in yeast and analyze strains for deficiencies in telomere maintenance.

***Task 5.*** Confirm that huStn1 and huTen1 are homologues of the yeast end protection complex (Months 24-28)

a. Create and characterize antibodies to huStn1 and huTen1.
b. Use immunofluorescence to determine cellular localization of huStn1 and huTen1.
c. Determine if huStn1, huTen1, and huPot1 exist in a complex by conducting co-immunoprecipitation and directed two-hybrid experiments.

***Task 6.*** Investigate the role of huStn1 and huTen1 in telomere maintenance. (Months 24-36)

a. Clone huStn1 and huTen1 cDNAs into appropriate inducible expression vectors.
b. Overexpress huStn1 and huTen1in human cell lines and analyze telomere length by standard Southern blot.
c. Construct dominant negative huStn1 ($huStn1^{DN}$) and huTen1 ($huTen1^{DN}$) expression vectors.
d. Express $huStn1^{DN}$ and $huTen1^{DN}$ and look for G-strand or C-strand degradation.
**e.** Use the cell lines created in Tasks 6e and 6g to investigate how huStn1 and huTen1 affect TRF2 telomeric localization.