Title
The telomeric cap and its regulation in budding yeasts

Author
Smith, Christopher David

Publication Date
2002

Peer reviewed|Thesis/dissertation

The Telomeric Cap and Its Regulation in Budding Yeasts

by

Christopher David Smith

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biochemistry and Molecular Biology

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

This thesis is dedicated to all of my parents, whose love and faith in me has always been a constant source of strength on this journey that was taken me so far away from them for so long.

Acknowledgements

It is hard to overstate how important so many people have been to me in the completion of this thesis. In the same way that biologists never *really* know *all* of the effects that their mutations and experimental perturbations have on living cells, my family, friends and colleagues surely cannot know how their words and actions have affected and enriched my life. I would not be the person I am today without them.

First and foremost, I would like to acknowledge Liz Blackburn, without whose guidance, perspective and relentless enthusiasm for my experiments, I would never have made it here today. Liz has taught me more about science, writing and myself than I ever thought possible. She has always given me the latitude to explore my other interests, even when they took me away from my bench. In spite of her considerable commitments both within and beyond the walls of UCSF, she has always taken the time to stop and talk about work, or nothing at all. She somehow always knew when words of encouragement were needed and when a manuscript needed a lot of red ink. Hence, there are few people who I am prouder to have worked with and to have learned from.

Irma Easter has made my stay at UCSF a far more pleasant one. Irma makes about everything we ever use for experiments and lots of it. She is hard working, very sweet even at 8am, and usually silent. I hate to think of how many stupid things I have said not knowing that she was right there, quietly filling our cabinets with everything we need to get things done. Thank you Irma, I will miss you.

Maura Devlin and Maura Clancy deserve unending praise for all the help they provided. Maura is actually only one person who just did the work of two. If it weren't

for her nobody in the lab would ever have known where Liz was, including Liz herself I imagine. I interrupted Maura far too often for little things I probably could have or should have figured out or done myself, but she always did everything she could to help me.

Laboratories are their own experiment in social interaction. Ironically, 10-15 people in a few rooms for six years has shown me more of the world than I ever might have learned from either reality television or a lot of expensive travel. Working side-by-side with researchers from Israel, India, China, "down under", and even other parts of the United States has given me a perspective on other countries and people that I will always cherish. I would like to thank all of the Blackburn Lab members past and present who have helped me to shape my ideas, perspectives and experiments and with whom I've had many hours of informative, colorful discussion about the world outside of lab. Without those many hours of talk about music, spyplanes, world politics, food & wine, gelato, or nothing in particular, I might have graduated a little sooner but certainly a lot less informed and entertained. In particular, I would like to thank my friend Anat Krauskopf for taking me under her wing when I first joined Liz's lab. John Prescott, Dudy Tzfati, Shivani Nautiyal, and Dana Smith have been particularly close friends who possessed the rare stamina to endure my bench blather. It goes without saying that this worked both ways.

My friends have also made my life in San Francisco and at UCSF truly wonderful. In particular, I need to thank the Verns. Jon Tupy taught me how to make a decent Manhattan and has been my most reliable camping partner for years now. Both of these things have made me a much happier person. Brandon Brown has provided me

with many welcome laughs and diversions as well. In the earlier days, before their diaspora around the country, Needhi Bhalla, Meghan Sharp, Oren Beske and Angela Newhoff were also some of my favorite people to spend time with. Finally, I need to acknowledge Jamie Marks, whose Élixir Végétal prepared me for the daily task of writing this thesis.

Sandy Johnson and Erin O'Shea were important mentors throughout graduate school. Both Sandy and Erin served on my qualifying exam committee as well as my thesis committee and have always pressed me to make my work the best that I can. Their down to earth advice helped me to sort out where I wanted to go from here and, more importantly, what I *really* needed to do to get there. Sandy has also always been a great source of camping information as well.

This thesis is dedicated to all of my parents, who I have missed very much since moving to California. Although my move saddened them, they never made me feel too guilty about it. For most of my life I have had two sets of parents, something that few people experience, and even fewer understand fully. Their support and advice has been indispensable to me, even more than when I relied on them completely for food, shelter, and everything else. Their confidence in my choices has sometimes served as my sole beacon that what I am doing is the right thing. For all of these things I am thankful.

Less abstractly, I never would have been able to fix the computers in my lab without my father, David Smith's help. I also had the pleasure of collaborating with him in the analysis of the microarray data described Chapter 3 and Appendix 4. Gail Hargrave-Smith has been flawless in her support of my every endeavor for many years.

Her calming perspective, compassion, and empathy have always helped center me. The yearly shipment of many boxes of girl scout cookies from her and my father helped me through the many rainy seasons. Francis Morbidelli has always given me an interested and untiring ear to tell about my camping trips and newfound trivia. Frank's sun-dried tomatoes have also made all of my friends jealous of me for as long as I can recall. There is no satisfactory way to thank my mother, JoAnn Morbidelli. In spite of hating every minute I have been away from New York, she, more than anyone, encouraged me to come to San Francisco because it was what I wanted. I am sure she only vaguely understands what I do, but somehow she has always made me feel like I am the best at it. Her many phone calls and grounding words have definitely helped me from losing sight of why I am here and not forgetting the real points of life.

Statement Regarding Previously Published Material with Multiple Authors

Chapter 2 has been previously published as "Uncapping and deregulation of telomeres lead to detrimental cellular consequences in yeast. Christopher D. Smith and Elizabeth H. Blackburn. Journal of Cell Biology Volume 145 Number 2, pp. 203-14". It is reprinted with permission from The Rockefeller University Press who retains all copyright privileges. The permission to reprint this material is provided in the following pages. I performed all of the experiments and co-authored the manuscript with Elizabeth Blackburn.

Chapter 3 is in the process of submission. The version included in this thesis may differ from the finally published version. I performed all of the experiments except for chromatin spread assays, which were performed with Dana Smith, whom also performed most of the budding indices. Joseph Derisi directed microarray printing and was essential for microarray analysis. I co-authored the text of the manuscript with Elizabeth Blackburn.

The remaining Chapters and Appendices were written entirely by me, and I performed all of the experiments described in these sections. However, the bank of putative *Kluyveromyces lactis* CDC mutants described in Appendix 3 was generated by Linda Silveira at the University of Redlands.

Elizabeth H. Blackburn, the last coauthor listed on each publication, directed and supervised the research that forms the basis of this dissertation.

UCSF
University of California
San Francisco

>chemistry and
>physics
abeth Blackburn, Ph.D.
fessor
Parnassus Avenue,
Francisco, CA
43-0448

415/476-4912
: 415/514-2913
>mer@itsa.ucsf.edu

November 15, 2001

The Journal of Cell Biology
Permissions
The Rockefeller University Press
1114 First Avenue
New York, NY 10021-8325

Letter via fax: 212-327-~~8025~~ 8589

To Whom It May Concern:

I write to request permission to reprint the manuscript and figures from the following publication for use in my Ph.D. thesis at the University of California, San Francisco:

Smith, C.D. and E.H. Blackburn Uncapping and deregulation of telomeres lead to detrimental cellular consequences in yeast. Journal of Cell Biology **145**(2): 203–214 (1999).

I am the first author on this manuscript.

Thank you.

Sincerely,

Christopher D. Smith
Elizabeth Blackburn Laboratory
University of California, San Francisco
513 Parnassus Avenue, Room S-469
San Francisco, CA 94143-0448
Tel: 415-476-2824
Fax: 415-514-2913

The Rockefeller
University Press
1114 First Avenue, 4th Floor
New York, New York 10021
(212) 327-7938
Fax (212) 327-8587

FAXED

November 29, 2001

Dr. Christopher D. Smith
Elizabeth Blackburn Laboratory
University of California, San Francisco
513 Parnassus Avenue, Room S-469
San Francisco, CA 94143-0448

Dear Dr. Smith:

We will grant you permission for the print reproduction of JCB-vol:145(2),203-214,1999-article as referred to in your letter dated November 15, 2001.

Permission is granted for one time use only. Please write to us each time for permission concerning future editions and translations, as we do not grant blanket permissions.

Since you are the author, a credit line is our only requirement.

Please give suitable acknowledgment to the source in the following manner: Reproduced from **The Journal of Cell Biology, year, vol., pp.** by copyright permission of The Rockefeller University Press.

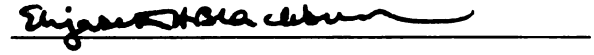
Sincerely yours,

THE JOURNAL OF
CELL BIOLOGY

Lynette N. Henry
Permissions Coordinator

Abstract

Eukaryotes hold their genetic information on linear chromosomes, whose stability is dependent upon specialized chromatin structures at the ends called telomeres. Telomeres prevent chromosome loss, fusion and uncontrolled recombination at the ends. In most organisms, the ability to regulate telomere size around a constant length is important for their function. When telomeres become too short they lose their ability to effectively protect the chromosome end and the cells may senesce. Likewise, when mutated telomeres become both elongated and deregulated, they may be less effective at protecting chromosome ends. In budding yeasts, cells with elongated and deregulated telomeres show morphological defects and evidence of polyploidy. Strikingly, the addition of a "cap" of only a few wild-type telomeric repeats is sufficient for cells to re-establish the ability to regulate their telomeres. Such cells are almost indistinguishable from wild-type, suggesting that extreme telomere length is itself is not detrimental to the cell. Three proteins that are thought to minimally comprise the telomeric chromatin cap, Rap1p, Rif1p and Rif2p, negatively regulate telomere length by inhibiting telomerase. We provide evidence for the dynamic remodeling of repressive telomeric chromatin that is correlated with the modification of Rif2p through the cell cycle. We also demonstrate that Rif1p is spread over many kilobases of telomeric chromatin, like Rap1 and the Sir proteins, and may be involved in long-range interactions between the terminal telomeric tracts and internal nucleosomal chromatin. The association of these repressive factors is lowest at points in the cell cycle when telomere addition occurs. Surprisingly, although the core telomerase protein Est2p remains associated with telomeric DNA throughout the cell cycle, it only adds telomeric DNA to the chromosome ends in mid-late S-phase.



Elizabeth H. Blackburn, Thesis Advisor

Table of Contents

Chapter 1

General Introduction

Trust your instinct to the end, though you can render no reason

Ralph Waldo Emerson (1803 - 1882)

Preface

Telomeres are protein-DNA complexes that protect the ends of linear chromosomes and are part of the elaborate cellular machinery that help to keep the genome stable. Telomeres have been likened to the plastic caps that keep shoelaces from unraveling and becoming unusable (Figure 1, compare A to C). Although we have long known that the telomeres, or literally the "end part" of chromosomes, are functionally different from the other parts of the chromosome, it has taken many years to determine *what* telomeres are made of and *how* their components are assembled into a structure that contributes to genome stability in living cells. The work encompassed by this thesis aims to address these topics.

The History and Background of Telomeres

In the late 19th century cytologists aided by views attained using clear, new, high-power microscopes, developed chemical stains and procedures to visualize the inner workings of cells. One thing they saw were rod-shaped structures located within the nucleus of eukaryotic cells. These structures were termed chromosomes (literally "colored body") and their substituent parts were divided loosely into the centromere, or center part, and the chromomere, which referred to any part or feature of interest along the length of the chromosome. Early on, researchers such as Karl Rabl noted that there were multiple chromosomes, they were linear with clearly visible ends, and they appeared to have reproducible orientations in the nucleus. Specifically, in 1885 Rabl

reported that he saw the chromosome ends were often clustered toward one side of the nucleus and sometimes appeared linked to the nuclear envelope (Rabl 1885). Even though Rabl did not know that the chromosomes he observed were the informational molecules of life, his observations are the earliest known where it became clear that eukaryotic chromosomes were linear with discrete ends and that those ends clustered and associated with the nuclear periphery.

In 1927 Hermann J. Muller began studying the effects of X-rays on the cytology of chromosomes in the common fruit fly *Drosophila*. He observed that irradiation led to gross chromosomal rearrangements such as inversions, translocations, and deletions of the internal regions, but never *terminal* deficiencies or inversions. In 1938, based on his cytological work in *Drosophila*, Muller and his contemporaries termed the end of the chromosomes as telomeres and he hypothesized that they "have a special function, that of sealing the end of the chromosome... and that for some reason a chromosome cannot persist indefinitely without having its ends thus 'sealed'" (Muller 1938). Although based on negative results, Muller's conclusions proved correct and are first illustration of how telomeres might prevent chromosome loss.

In 1941, experiments carried out by Barbara McClintock further elucidated the importance of telomeres in chromosome stability. In maize, she showed that broken chromosome ends were "sticky" and fused together. Furthermore, she showed that when cells containing these fusions tried to divide, the end result was a breakage of the chromosome end. Strikingly, these broken ends would fuse again in the next cell cycle and continue the breakage-fusion cycle indefinitely (McClintock 1941). Thus,

McClintock's work showed that another function of the telomere was to prevent the fusion of chromosome ends.

Once DNA was established as the repository of genetic information and its structure had been solved (reprinted as Watson and Crick 1974), theoretical speculation about the physical structure of telomeres and the mechanism of their replication progressed. In the early 1970's Watson and Olovnikov theorized that one end of a linear chromosome could not be fully replicated by conventional DNA polymerases (Watson 1972; Olovnikov 1973). They proposed that one strand of synthesized DNA would always have a short stretch that could not be fully copied due to the need for a RNA primer to initiate DNA polymerase. The final outcome of "the end replication problem" was proposed to be gradual erosion of the chromosome end (i.e. telomeric) region and eventually loss of the genetic information interior to chromosome ends. These were the first published speculations that a specialized mechanism for maintaining chromosome ends might exist.

A number of theories about how to avoid the end replication problem emerged. Concatamerization was suggested as one method to replicate the ends (Watson 1972). Other researchers suggested that DNA might contain palindromic sequences that folded over and allowed the end to "self-prime" (Cavalier-Smith 1974). The breakthrough in solving the end replication problem began in 1978 when Elizabeth Blackburn and Joseph Gall presented findings that characterized the DNA sequence of the ends of linear chromosomes from the ciliated protozoa *Tetrahymena thermophila* (Blackburn and Gall 1978). The telomeric sequence from *Tetrahymena* consisted of tandem TG-rich hexameric repeats (5'- TTGGGG) on the chromosome ends (Blackburn and Gall 1978).

The identification of similar sequences from other ciliates and several other species suggested that although telomeric DNA sequence was not absolutely conserved between organisms, it generally consisted of G-rich repeats (Klobutcher, Swanton et al. 1981; Szostak and Blackburn 1982; Shippen-Lentz and Blackburn 1990), reviewed in (Henderson 1995). Strikingly, telomeric DNA from ciliates functioned when introduced into the budding yeast *Saccharomyces cerevisiae*, suggesting that the fundamental properties of telomeric DNA were conserved through a wide range of eukaryotes (Shampay, Szostak et al. 1984). Another basic and important observation made of telomeres in most organisms was that their lengths fell in a restricted range that was tightly regulated and species specific. This was first observed in 1978 when Blackburn and Gall saw that telomeric fragments were heterogeneous in size on agarose gels but nonetheless constricted within a defined length range. This phenomenon, referred to as telomere length regulation, has been observed in most eukaryotes. For example, certain ciliate telomeres are generally less than 100 base pairs (bp), while those of *Tetrahymena* and *S. cerevisiae* range from 300-350 bp, and human telomeres can vary between 5 – 25 kB, depending on the cell type. Although Blackburn's work had not yet demonstrated the mechanism of telomere addition, it did show that specialized DNA was deposited at the telomeres. From her work three important properties about telomeres were also shown: telomeric sequence was G-rich, composed of repetitive sequences, and the total length of telomeric repeats was somehow regulated and confined to a narrow range.

Telomerase Emerges as a Specialized Molecule for Telomere Maintenance

In 1985 Carol Greider and Elizabeth Blackburn identified telomerase, the biochemical activity that adds telomeric DNA to the ends of chromosomes (Greider and Blackburn 1985). The discovery of telomerase as the activity that added specialized DNA to the chromosome end obviously heralded a new age in telomere research. While initially termed to be a terminal transferase protein, telomerase was soon identified as a specialized type of ribonucleoprotein that used an RNA template to copy DNA telomeric repeats onto chromosome ends (Greider and Blackburn 1987; Greider and Blackburn 1989; Shippen-Lentz and Blackburn 1990). Until the discovery of the telomerase RNA, it had been thought that reverse transcription was a mode of replication used only by viruses and transposons.

Using newly discovered telomeric repeat sequences as a probes to hybridize to genomic DNA libraries, the gene encoding the telomerase RNA, generically referred to in all species as *TER1* (reviewed in (Henderson 1995)), was cloned first from ciliates and thereafter from many other organisms, including *S. cerevisiae* (called *TLC1)* and humans (also called hTR). While the identification of *TER1* genes did greatly facilitate research within the model system that they were cloned out of, the low homology of RNA species between even closely related species made it difficult to find conserved structures or related molecules in other model systems. Unlike the ribosomal RNA genes, which show regions of broad conservation between all living things, telomerase RNA genes are quite

divergent at the sequence level. Telomerase RNA molecules can also vary widely in size, depending upon the organism they were cloned from. For example, ciliates have small *TER1* genes (~150-200 bp) (Greider and Blackburn 1989; Shippen-Lentz and Blackburn 1990), while the human *TER1* gene, hTR, is ~450 bp (Feng, Funk et al. 1995). The *TER1* genes from budding yeasts are typically greater than 1 kB in size (McEachern and Blackburn 1994; Singer and Gottschling 1994). Despite their heterogeneity in size, all telomerase RNA genes perform the same function, providing a short template sequence for telomeric repeats. However, only a small part of telomerase RNA contains the short region that serves as the template for adding telomeric DNA to the chromosome end. The rest of the telomerase RNA contains some conserved sequences and also forms hairpins, stem-loops, and pseudo-knots that presumably give telomerase structure by aiding interaction with the core protein component of telomerase (TERT/Est2p)(Bryan, Goodrich et al. 2000) or possibly other telomerase cofactors (see below). Mutations in *TER1* that allow TERT binding can also cause loss of enzymatic activity or result in aberrant catalytic activity (Gilley, Lee et al. 1995; Gilley and Blackburn 1996; Prescott and Blackburn 1997; Licht and Collins 1999). Only recently have conserved regions in the telomerase RNA gene been identified in yeast, ciliates, and vertebrates (Romero and Blackburn 1991; Bhattacharyya and Blackburn 1994; Lingner, Hendrick et al. 1994; Blasco, Funk et al. 1995; Chen, Blasco et al. 2000; Tzfati, Fulton et al. 2000; Sperger and Cech 2001). Evidence of essential stem-loop and pseudoknot structures in the telomerase RNAs of yeast (Yehuda Tzfati, personal communication), ciliates (ten Dam, van Belkum et al. 1991; Gilley and Blackburn 1999), and humans (Bachand, Triki et al. 2001), makes

it likely that some basic structures and functions have been conserved between the divergent RNAs of these species.

The identification of the protein component of telomerase finally occurred over a decade after the purification of telomerase activity from ciliates. While the initial biochemical identification of telomerase and telomere proteins in ciliates seemed promising, their use of an alternate genetic code made artificial protein expression and sequence comparison to "conventionally-coded" genes in other species cumbersome. The isolation of the telomerase associated proteins p80 and p95 in ciliates raised hopes that a widely conserved catalytic protein core of telomerase existed (Collins, Kobayashi et al. 1995). Although p80 co-purifies with telomerase activity, contains RNA binding activity (Gandhi and Collins 1998), and has mammalian homologs (Harrington, McPhail et al. 1997; Nakayama, Saito et al. 1997), neither it nor p95 are required for reconstituted telomerase activity (Mason, Autexier et al. 2001). Instead p80 and p95 are likely to be cofactors required for the assembly, recruitment, or processivity of telomerase and indicate that telomerase, like other polymerases, may be behave more like a complex, regulated holoenzyme instead of a simple biochemical activity. It took the simultaneous identification of the putative telomerase protein from multiple model organisms to uncover their relationships to one another and reverse transcriptases in general. The breakthrough came when tandem mass spectroscopic analysis of *Euplotes aediculatus* telomerase activity revealed a 123 kD protein that had homology to the previously identified *S. cerevisiae* gene, *EST2*, which is essential for telomerase function (Lendvay, Morris et al. 1996; Lingner, Hughes et al. 1997). Soon after, a human *EST2* homolog was discovered by homology in the human genome database, and it was realized that all

telomerase catalytic proteins contained conserved reverse transcriptase domains (Lingner, Hughes et al. 1997; Meyerson, Counter et al. 1997).

Telomerase has not been identified in *Drosophila* to date. Instead, fruit flies use highly repetitive retrotransposons to maintain the integrity of the chromosome ends (Biessmann, Valgeirsdottir et al. 1992). Retrotransposons are a distinct subclass of transposons that require a reverse-transcription step to propagate themselves. Despite the apparently radical differences between these two modes of telomere replication, they are actually quite similar. In the case of telomerase-type telomere replication, only the telomerase RNA template is reverse transcribed onto the chromosome end, while in the case of *Drosophila* retrotransposons, the whole RNA molecule is reverse transcribed onto the chromosome end. Thus, in the most basic sense, even telomerase-less organisms use some sort of reverse transcription process to maintain telomeres.

Telomeres and Telomerase are Implicated in Cellular Lifespan and Cancer

Around the time that the DNA sequence of human telomeric repeats was identified (Moyzis, Buckingham et al. 1988), telomerase activity was identified in HeLa cells (Morin 1989). Shortly thereafter it was found that the telomere length of fibroblasts became progressively shorter as the cells were passaged in culture (Harley, Futcher et al. 1990). It had already established that cultured fibroblasts were limited to about 50 population doublings, with an increasing fraction of the population exiting the cell cycle and causing the population as a whole eventually to undergo cellular senescence

(Hayflick 1979; Pontaen 1983). Since such cells were shown to lack telomerase activity, the notion that the shortening of telomeres thus acted like a "molecular clock" that limited the division potential of cells quickly gained acceptance. It has since been discovered that many other normal cell types undergo a similar phenomenon and a host of other data has further substantiated this hypothesis. First, germ line cells, which are passed on between generations, do not exhibit telomere shortening and have detectable levels of telomerase activity. Stem cells and other self-renewing cells such the skin and immune system also exhibit telomerase activity. Similarly, unicellular organisms, such as ciliates and yeasts, which divide indefinitely, also have detectable telomerase activity and stable telomere lengths. On the other hand, somatic cells generally, which do not have telomerase activity, do exhibit telomere shortening and eventual cellular senesce. The interpretation of these observations is that cells that continually divide require telomerase for viability, while cells that do not actively divide can subsist on their existing pool of telomeric repeats.

In a landmark experiment, the overexpression of telomerase components was shown to extend cellular lifespan of cultured cells indefinitely, strongly suggesting that telomere length and/or telomerase activity were important prognosticators of cellular replicative potential (Bodnar, Ouellette et al. 1998). The frequent activation of telomerase in cancer cells supports this interpretation. One simple definition of cancer cells is that they are cells that develop detrimental genomic lesions (transformation) and proliferate out of control (immortalization), resulting in the rapid clonal expansion of transformed cells (i.e. tumors). In theory, without the activation of telomerase, transformed cells would quickly run into the Hayflick limit and senesce. Cells that had

not experienced a transforming event would be predicted to simply divide indefinitely, as is observed with germ-line cells. The high levels of telomerase activity common in human cancers suggests that the activation of telomerase is a prerequisite that allows transformed cells to continue dividing (Meyerson, Counter et al. 1997). Since one hallmark of cancer cells is genomic instability, it has been historically difficult to define the precise genetic requirements for cancer. However, the creation of tumor cells with defined genetic elements demonstrated that telomerase activation is a necessary step to immortalize cells that had already been transformed by defined viral oncogenic genes (Hahn, Counter et al. 1999).

The correlation of telomere length, cellular replicative potential, and the activation of telomerase in many cancers unified our understanding of cellular lifespan and the importance of telomeres in genomic stability. These experiments hypothesized the fundamental importance of telomerase in human disease and the mechanisms that regulate or control telomere length have since been intensely studied in many model systems.

Budding Yeast, A Model System to Study the Genetics of Telomere Biology

The discovery that the majority of eukaryotic organisms maintain their telomeres by the RNP telomerase, or some other reverse transcription process, suggests that it is an ancient, widely conserved mode of DNA end replication. The discovery that telomerase plays a pivotal role in cellular lifespan and cancer has further shown the importance of this basic mechanism in the health of cells. On one hand, this knowledge has given us

the perspective to look carefully for the fundamental similarities between the telomere maintenance systems of distantly related organisms. On the other hand, the genes that encode components of the telomere maintenance system have significantly diverged in sequence, making the standard method of cloning and comparing proteins or RNAs in other species by homology difficult. However, as in-depth analysis of the telomerase RNA and catalytic subunit genes have shown, many fundamental aspects of telomere genes are conserved across species, including those that, like *Drosophila*, do not even use telomerase.

After the identification of the genes that encode the core telomerase activity, it became easier to expand telomere research into other model systems. While ciliates have the biochemical advantage of tens of thousands to millions of chromosome ends to purify telomerase from, they lack simple tools to easily knock out and add back genes that might be involved in the pathways of telomere maintenance. Likewise, while the links between telomerase, cellular aging, and cancer were most developed in human model systems, the difficulty of human genetics and the slow growth of cultured cells made genetic analysis difficult. Adaptation of biochemical approaches used in the identification of ciliate telomere proteins and the application of the principles learned from human model systems coupled with the power of yeast genetics has facilitated the identification of numerous genes that affect telomere biology in *S. cerevisiae*. Numerous factors have made budding yeast a "model" model system for telomere biology. The history of genetic manipulation, relatively small and completely sequenced genome, and ease of mutagenesis, and amenability to biochemical studies make yeast an advantageous system to explore the genetics of telomere biology.

Since the proper functioning of telomeres is often directly correlated with their length, telomere size is an important readout of the effects of genes on telomere maintenance. Any gene that has dramatic, or reproducibly subtle, effects on telomere length or its regulation can be considered a part of the homeostatic system that keeps telomere length regulated in a defined, tight range. There are currently over 50 genes that have been implicated in telomere maintenance in yeast. These genes and a brief description of their role in telomere biology are listed in Table 1. Several of the more central factors in telomere length control will be discussed below.

In order to understand the various proteins that are involved in telomere length regulation, it is necessary to understand the topology of telomeric DNA itself. Telomeric DNA is predominantly double stranded. As previously mentioned, the length of the double-stranded telomere tract varies widely between species, but is confined to a narrow range within any given cell type. The G-rich strand is templated and synthesized by telomerase and the conventional DNA replication machinery copies the complementary C-rich strand. Telomeres in most organisms have a short G-strand overhang at the distal tip of the chromosome (Klobutcher, Swanton et al. 1981; Pluta, Kaine et al. 1982; Henderson and Blackburn 1989; Wellinger, Wolf et al. 1993; McElligott and Wellinger 1997). Recent evidence in mammalian systems suggests that this single stranded region may actually interact with the double-stranded telomeric region to form a specialized triple-stranded "T-loop" structure (Griffith, Comeau et al. 1999).

In most organisms studies there exist DNA binding proteins that interact specifically with either the double-stranded or single stranded telomeric DNA. Proteins that bind to telomeric DNA fall into two categories: factors that promote telomerase

addition to the chromosome end (positive regulators) and ones that inhibit telomerase (negative regulators). For example, in ciliates, the α and β proteins dimerize in the presence of single stranded telomeric DNA and bind to it (Gottschling and Cech 1984). These proteins function to prevent telomerase elongation *in vitro*, presumably by occupying its substrate at the chromosome end, and, therefore, can be considered negative regulators of telomere length. In *S. cerevisiae*, Cdc13p (*EST4*) binds to single stranded telomeric DNA. Cdc13p has been shown to recruit telomerase to the chromosome end through the Est1p adapter protein (Evans and Lundblad 1999). Thus, Cdc13p is said to be a positive regulator of telomere length. Interestingly, the Cdc13p interacting protein Stn1p can also associate with Cdc13 and thereby inhibit telomerase. Thus, telomeric proteins may have multiple functions that are dependent upon the binding partners available in that context (Pennock, Buckley et al. 2001).

Since the majority of telomeric DNA is double stranded, proteins that bind to double stranded DNA are often the most abundant at the chromosome end. Generally, proteins that bind to the double stranded telomeric regions are thought of as "negative regulators" of telomerase because they recruit other proteins to create a non-nucleosomal chromatin that is thought to inhibit the access of telomerase at the chromosome end (discussed in more depth below). For example, the major telomere binding protein in yeast is the Repressor Activator Protein, Rap1p (Buchman, Kimmerly et al. 1988). Other proteins interact with Rap1p via the protein's C-terminus, resulting in a protein complex that protects the chromosome ends. In mammalian cells, the major telomere binding proteins are hTRF1 (Zhong, Shiue et al. 1992)and hTRF2 (Bilaud, Brun et al. 1997). The general principle that holds true between the major double-strand telomere binding

proteins of all organisms is that they interact with their telomere sequence through Myb domains located in the conserved DNA binding domain of the protein (Broccoli, Smogorzewska et al. 1997). Although telomere binding proteins all seem to have or recruit a repressive telomeric complex that inhibits telomerase, their specific binding partners are variable between groups of organisms.

Positive Regulators of Telomerase activity in Yeast

The positive regulators of telomere length are genes that promote the addition of telomeric DNA. These might include the genes for the telomerase enzyme and its cofactors or molecules in the putative signaling pathway that "senses" length and directs telomerase to begin and continue addition when needed. When genes for positive regulators of telomerase are ablated or mutationally compromised, the telomeres generally shorten in length. If the gene is essential for telomere maintenance, as opposed to a non-essential regulator of the process, the telomeres will shorten to the point of senescence and the cell will die unless it finds an alternate way to maintain its telomeres.

The first genes recognized to be essential for telomere maintenance in yeast were the Ever-Shortening-Telomere (*EST1-4*) genes and the telomerase RNA gene *TLC1*. Est2p and *TLC1* comprise the protein catalytic core of telomerase. Cdc13p is the single stranded DNA binding protein encoded by the *EST4* gene and is thought to directly recruit telomerase to the chromosome end, via interaction with the telomerase subunit Est1p (Evans and Lundblad 1999; Pennock, Buckley et al. 2001). Stn1p and Ten1p, which interact with one another and Cdc13p, also play a role in telomerase recruitment and length regulation (Grandin, Damon et al. 2001; Pennock, Buckley et al. 2001). The

EST1 and *EST3* genes are required for telomerase function *in vivo*, but not for *in vitro* activity. The role of Est3p remains unclear (Hughes, Evans et al. 2000).

A number of others factors are required for telomere maintenance *in vivo*. Telomerase action also requires Polα and Pol∂, and is promoted by the *MRE11*, *RAD50*, *XRS2* (MRX) complex (Lendvay, Morris et al. 1996; Nugent, Bosco et al. 1998; Diede and Gottschling 1999; Ritchie and Petes 2000) and the Ku proteins (Peterson, Stellwagen et al. 2001). Polα, and Pol∂ are thought to coordinate telomere replication with the conventional DNA replication machinery (Diede and Gottschling 1999). The role of the MRX complex likely involves modulating the interaction between telomerase and its telomeric DNA substrate (Ritchie and Petes 2000; Diede and Gottschling 2001; Tsukamoto, Taggart et al. 2001). The Ku proteins are highly conserved in all eukaryotes and play a prominent role in the cell as mediators of non-homologous end joining and DNA damage response to double stranded breaks. The Ku proteins also interact with a stem loop of the RNA component of telomerase, *TLC1*, and Sir4p, and are required for the maintenance of silent chromatin at the telomeres (Tsukamoto, Kato et al. 1997; Boulton and Jackson 1998; Peterson, Stellwagen et al. 2001). While the mechanism remains unclear, Ku proteins are thought to distinguish the telomeric end from a double-strand break.

Other genes that are not necessarily localized on the telomeric DNA have also been implicated telomere maintenance. The *TEL1 & TEL2* genes were isolated as cell-cycle-defective alleles that resulted in telomere shortening (Lustig and Petes 1986). Tel1p is part of the highly conserved ATM family of kinases and is thought to be involved in the signal transduction processes that regulate telomere length through the

MRX complex (Greenwell, Kronmal et al. 1995). Although *TEL2* is an essential gene that binds to telomere DNA, its function is unknown (Runge and Zakian 1996). Many other genes with indirect effects on telomere have also been identified. Since telomere replication is linked to standard DNA replication, it is highly probable that other components essential for DNA synthesis, repair, and damage response are also required for proper telomere replication.

Negative Regulators of Telomerase activity in Yeast

The negative regulators of telomeres inhibit telomere lengthening. These genes include those that may inhibit the recruitment of telomerase or package its substrate into an inaccessible chromatin state. In addition, genes that inhibit telomere elongation or promote end degradation of the single stranded DNA are also technically negative regulators. Generally, when the negative regulators of telomere maintenance are mutated or knocked-out, the telomeres lengthen and lose their ability to regulate lengths around the proper length "set-point". The Repressor-Activator Protein, Rap1p, was biochemically identified as the major double-stranded telomere binding activity in *S. cerevisiae* and *K. lactis*. In addition to binding double stranded telomeric DNA, the *RAP1* gene product is responsible for transcriptionally activating and repressing genes involved in carbohydrate metabolism and ribosome synthesis as well binding the silent mating type loci. While there is a human homolog to scRAP1, it does not appear to bind telomeric DNA directly. Instead, hTRF2 tethers hRAP1, and potentially other proteins, in the same way that scRap1p tethers non-nucleosomal chromatin in yeast. In yeast, the C-terminal tail of Rap1p interacts with a number of factors that comprise the telomere-

specific chromatin, or telosome. The Silent Information Regulator, Sir3p protein interacts with Rap1p and may simultaneously interact with Sir4p or Histone H3 & H4 tails. Sir4p also interacts with Rap1p, the Ku protein, Sir2p, and with histone tails in nucleosomal DNA. While deletion of the *SIR3* or *SIR4* genes causes only slight shortening of the telomere, the Sir proteins are essential for the clustering of the telomeres and their attachment to the nuclear periphery. The Rap1p-Interacting Factors, Rif1p and Rif2p, were isolated as factors that interacted with Rap1p in a 2-hybrid screen. Like the Sir3p or Sir4p, Rif1p and Rif2p interact with the Rap1p- C-terminus. However, unlike the *SIR3* or *SIR4* genes, ablation of either of the *RIF* genes results in telomere lengthening and some loss of regulation, and deletion of both results in a synergistic loss of telomere length control. The Rif proteins interact with one another in addition to Rap1p. High throughput 2-hybrid studies suggest that Rif2p may have as many as 80 binding partners (Ito, Chiba et al. 2001).

The end result of the major factors that associate with Rap1p and further associate with one another is a non-nucleosomal complex that is strengthened by many redundant interactions. The Rap1-Rif-Sir complex further interacts with the tail of conventional nucleosomal histone tails and it though to fold over the chromosome end and protect it. This model of protective telomeric chromatin in yeast is reminiscent of telomeric chromatin structures called T-loops that have been directly visualized in mammalian cells (Griffith, Comeau et al. 1999). T-loops are hypothesized to form as a result of the G-rich overhang looping back and strand invading double-stranded telomeric DNA. This results in a closed loop at the chromosome end that is further bound by the protective TRF proteins. While T-loops have not been visualized in yeast, the mutually reinforcing

interactions between Rap1, Rifs, Sirs, and conventional nucleosomes are thought to achieve the same end, the looping back of the chromosome end into a higher order protective complex.

Telomere Length Homeostasis and the Rationale for this Project

The end result of the positive and negative regulators on telomere length maintenance is a dynamic balance of activities that lengthen telomeres and those that repress lengthening. The sum of these competing and balanced activities results in the generally regulated telomere length that is generally observed in most species. The mechanisms of how the cell regulates telomere length remain poorly understood. Perturbation of telomere length through mutation of the telomeric DNA sequence or the proteins that bind telomeric DNA have been the main tools used in studying the relationship between repressive telomeric chromatin and the positive telomere lengthening activities.

Around the time I began my thesis work, the *TER1* gene had been cloned from the related budding yeast, *Kluyveromyces lactis*, which had long, "perfectly" copied repeats that could incorporate restriction sites into the telomeres when mutagenized. I became interested in the cellular and genomic phenotypes of these strains whose telomeric DNA had been mutagenized. Normally *K. lactis* telomeres are 250-500 base pairs (10-20 tandem 25 bp repeats), but some strains containing mutant repeats had telomeres that were elongated over a broad size range (e.g. <0.5-25 kB vs. 250-500 bp). Moreover, strains with elongated, deregulated telomeres were often slow growing and had sick looking colony morphologies. Strikingly, the degree of telomere lengthening

was sometimes, but not always correlated with Rap1p binding, which had already been well established as the major telomere binding protein in yeasts.

At the time when I began my thesis project, *S. cerevisiae* had the advantage of many identified genes and a genome that was nearly sequenced. However, the only mutations that dramatically affected telomere length were C-terminal truncations in Rap1p that destroyed its ability to bind with any of its protein partners. Unfortunately, *S. cerevisiae* telomeric repeats are not exact tandem repeats but "degenerate" G-rich repeats, making the reliable incorporation of altered telomeric repeats into the telomere difficult. Thus, it was hard to ascertain when mutant repeats were incorporated in the telomeres. Additionally, *S. cerevisiae* contains considerable amounts of internal telomeric repeats, which makes Southern blot analysis complex compared to *K. lactis*. For these reasons, there had not been extensive mutagenesis of the *S. cerevisiae* telomere sequence and the tools to study the effects of telomeric sequence on the RAP1 complex did not exist. It was already known that many genes complemented between *K. lactis* and *S. cerevisiae*. I therefore decided to exploit the experimental advantages of the *K. lactis* system and begin exploring the roles of telomeric DNA sequences on its length regulation mechanism, hoping that any mechanisms I uncovered could be easily applied to *S. cerevisiae*.

In Chapter 2 of this thesis, I investigate the effects of mutagenized telomeric repeats in the budding yeast *K. lactis*. Repeats that result in the eventual elongation and re-regulation of telomeric DNA are correlated with arrested, multi-budded cells, which have increased DNA content. This phenotype does not correlate the ability of Rap1p to bind the telomeric repeat, since two mutants examined have no Rap1p binding defect *in vitro*. Strikingly, the addition of only a few wild type repeats allows these sick cells to

recover, still with their elongated telomeres. Appendix 1 investigates the differences of DNA bending in different mutant template repeats. In appendix 2, timecourse experiments analyzing *K. lactis* cellular senescence after telomerase RNA deletion are presented. Appendix 3 describes the screening of a temperature-sensitive library of putative CDC mutants that was generated by Linda Silveira.

After studying the dramatic ability of short stretches of WT telomeric DNA to effectively "cap" and re-regulate the chromosome end in *K. lactis*, I became interested in the molecular nature of telomeric chromatin and how it functioned. The identification of many factors involved in telomere biology that are also involved in other cellular processes, such as DNA replication, chromatin remodeling and DNA damage, suggested that association of proteins to the chromosome ends was probably regulated. I became interested in whether chromatin remodeling happened at the telomere and what factors might be involved. I worked from the basic premise that different proteins must be associated depending on whether a telomere is being replicated or protected. Understanding the dynamic way in which proteins associate and dissociate from the telomeric complex over the cell cycle during is the topic of Chapter 3. Specifically, I describe experiments investigating the associations of Rap1p, Rif1p, Rif2p, and Est2p with telomeric DNA throughout he cell cycle. From these results, I offer a more in depth model of how telomeric chromatin changes to meet the needs of cell at different stages of the cell cycle.

References

Bachand, F., I. Triki, et al. (2001). "Human telomerase RNA-protein interactions." Nucleic Acids Res **29**(16): 3385-93.

Bhattacharyya, A. and E. H. Blackburn (1994). "Architecture of telomerase RNA." Embo J **13**(23): 5721-3.

Biessmann, H., K. Valgeirsdottir, et al. (1992). "HeT-A, a transposable element specifically involved in "healing" broken chromosome ends in Drosophila melanogaster." Mol Cell Biol **12**(9): 3910-8.

Bilaud, T., C. Brun, et al. (1997). "Telomeric localization of TRF2, a novel human telobox protein." Nat Genet **17**(2): 236-9.

Blackburn, E. H. and J. G. Gall (1978). "A tandemly repeated sequence at the termini of the extrachromosomal ribosomal RNA genes in Tetrahymena." J Mol Biol **120**(1): 33-53.

Blasco, M. A., W. Funk, et al. (1995). "Functional characterization and developmental regulation of mouse telomerase RNA." Science **269**(5228): 1267-70.

Bodnar, A. G., M. Ouellette, et al. (1998). "Extension of life-span by introduction of telomerase into normal human cells." Science **279**(5349): 349-52.

Boulton, S. J. and S. P. Jackson (1998). "Components of the Ku-dependent non-homologous end-joining pathway are involved in telomeric length maintenance and telomeric silencing." Embo J **17**(6): 1819-28.

Broccoli, D., A. Smogorzewska, et al. (1997). "Human telomeres contain two distinct Myb-related proteins, TRF1 and TRF2." Nat Genet **17**(2): 231-5.

Bryan, T. M., K. J. Goodrich, et al. (2000). "Telomerase RNA bound by protein motifs specific to telomerase reverse transcriptase." Mol Cell **6**(2): 493-9.

Buchman, A. R., W. J. Kimmerly, et al. (1988). "Two DNA-binding factors recognize specific sequences at silencers, upstream activating sequences, autonomously replicating sequences, and telomeres in Saccharomyces cerevisiae." Mol Cell Biol **8**(1): 210-25.

Cavalier-Smith, T. (1974). "Palindromic base sequences and replication of eukaryote chromosome ends." Nature **250**: 467-70.

Chen, J. L., M. A. Blasco, et al. (2000). "Secondary structure of vertebrate telomerase RNA." Cell **100**(5): 503-14.

Collins, K., R. Kobayashi, et al. (1995). "Purification of Tetrahymena telomerase and cloning of genes encoding the two protein components of the enzyme." Cell **81**(5): 677-86.

Diede, S. J. and D. E. Gottschling (1999). "Telomerase-mediated telomere addition in vivo requires DNA primase and DNA polymerases alpha and delta." Cell **99**(7): 723-33.

Diede, S. J. and D. E. Gottschling (2001). "Exonuclease activity is required for sequence addition and Cdc13p loading at a de novo telomere." Curr Biol **11**(17): 1336-40.

Evans, S. K. and V. Lundblad (1999). "Est1 and Cdc13 as comediators of telomerase access." Science **286**(5437): 117-20.

Feng, J., W. D. Funk, et al. (1995). "The RNA component of human telomerase." Science **269**(5228): 1236-41.

Gandhi, L. and K. Collins (1998). "Interaction of recombinant Tetrahymena telomerase proteins p80 and p95 with telomerase RNA and telomeric DNA substrates." Genes Dev **12**(5): 721-33.

Gilley, D. and E. H. Blackburn (1996). "Specific RNA residue interactions required for enzymatic functions of Tetrahymena telomerase." Mol Cell Biol **16**(1): 66-75.

Gilley, D. and E. H. Blackburn (1999). "The telomerase RNA pseudoknot is critical for the stable assembly of a catalytically active ribonucleoprotein." Proc Natl Acad Sci U S A **96**(12): 6621-5.

Gilley, D., M. S. Lee, et al. (1995). "Altering specific telomerase RNA template residues affects active site function." Genes Dev **9**(18): 2214-26.

Gottschling, D. E. and T. R. Cech (1984). "Chromatin structure of the molecular ends of Oxytricha macronuclear DNA: phased nucleosomes and a telomeric complex." Cell **38**(2): 501-10.

Grandin, N., C. Damon, et al. (2001). "Ten1 functions in telomere end protection and length regulation in association with Stn1 and Cdc13." Embo J **20**(5): 1173-83.

Greenwell, P. W., S. L. Kronmal, et al. (1995). "TEL1, a gene involved in controlling telomere length in S. cerevisiae, is homologous to the human ataxia telangiectasia gene." Cell **82**(5): 823-9.

Greider, C. W. and E. H. Blackburn (1985). "Identification of a specific telomere terminal transferase activity in Tetrahymena extracts." Cell **43**(2 Pt 1): 405-13.

Greider, C. W. and E. H. Blackburn (1987). "The telomere terminal transferase of Tetrahymena is a ribonucleoprotein enzyme with two kinds of primer specificity." Cell **51**(6): 887-98.

Greider, C. W. and E. H. Blackburn (1989). "A telomeric sequence in the RNA of Tetrahymena telomerase required for telomere repeat synthesis." Nature **337**(6205): 331-7.

Griffith, J. D., L. Comeau, et al. (1999). "Mammalian telomeres end in a large duplex loop." Cell **97**(4): 503-14.

Hahn, W. C., C. M. Counter, et al. (1999). "Creation of human tumour cells with defined genetic elements." Nature **400**(6743): 464-8.

Harley, C. B., A. B. Futcher, et al. (1990). "Telomeres shorten during ageing of human fibroblasts." Nature **345**(6274): 458-60.

Harrington, L., T. McPhail, et al. (1997). "A mammalian telomerase-associated protein." Science **275**(5302): 973-7.

Hayflick, L. (1979). "The cell biology of aging." J Invest Dermatol **73**(1): 8-14.

Henderson, E. (1995). Telomere DNA Structure. Telomeres. E. H. B. a. C. W. Greider. Cold Spring Harbor, Cold Spring Harbor Press, NY: 11-34.

Henderson, E. R. and E. H. Blackburn (1989). "An overhanging 3' terminus is a conserved feature of telomeres." Mol Cell Biol **9**(1): 345-8.

Hughes, T. R., S. K. Evans, et al. (2000). "The Est3 protein is a subunit of yeast telomerase." Curr Biol **10**(13): 809-12.

Ito, T., T. Chiba, et al. (2001). "A comprehensive two-hybrid analysis to explore the yeast protein interactome." Proc Natl Acad Sci U S A **98**(8): 4569-74.

Klobutcher, L. A., M. T. Swanton, et al. (1981). "All gene-sized DNA molecules in four species of hypotrichs have the same terminal sequence and an unusual 3' terminus." Proc Natl Acad Sci U S A **78**(5): 3015-9.

Lendvay, T. S., D. K. Morris, et al. (1996). "Senescence mutants of Saccharomyces cerevisiae with a defect in telomere replication identify three additional EST genes." Genetics **144**(4): 1399-412.

Licht, J. D. and K. Collins (1999). "Telomerase RNA function in recombinant Tetrahymena telomerase." Genes Dev **13**(9): 1116-25.

Lingner, J., L. L. Hendrick, et al. (1994). "Telomerase RNAs of different ciliates have a common secondary structure and a permuted template." Genes Dev **8**(16): 1984-98.

Lingner, J., T. R. Hughes, et al. (1997). "Reverse transcriptase motifs in the catalytic subunit of telomerase." Science **276**(5312): 561-7.

Lustig, A. J. and T. D. Petes (1986). "Identification of yeast mutants with altered telomere structure." Proc Natl Acad Sci U S A **83**(5): 1398-402.

Mason, D. X., C. Autexier, et al. (2001). "Tetrahymena proteins p80 and p95 are not core telomerase components." Proc Natl Acad Sci U S A **98**(22): 12368-73.

McClintock, B. (1941). "The stability of broken ends of chromosomes in *Zea Mays*." Genetics **26**: 234-82.

McEachern, M. J. and E. H. Blackburn (1994). "A conserved sequence motif within the exceptionally diverse telomeric sequences of budding yeasts." Proc Natl Acad Sci U S A **91**(8): 3453-7.

McElligott, R. and R. J. Wellinger (1997). "The terminal DNA structure of mammalian chromosomes." Embo J **16**(12): 3705-14.

Meyerson, M., C. M. Counter, et al. (1997). "hEST2, the putative human telomerase catalytic subunit gene, is up- regulated in tumor cells and during immortalization." Cell **90**(4): 785-95.

Morin, G. B. (1989). "The human telomere terminal transferase enzyme is a ribonucleoprotein that synthesizes TTAGGG repeats." Cell **59**(3): 521-9.

Moyzis, R. K., J. M. Buckingham, et al. (1988). "A highly conserved repetitive DNA sequence, (TTAGGG)n, present at the telomeres of human chromosomes." Proc Natl Acad Sci U S A **85**(18): 6622-6.

Muller, H. J. (1938). "The remaking of chromosomes." Collecting Net **8**: 182-95,198.

Nakayama, J., M. Saito, et al. (1997). "TLP1: a gene encoding a protein component of mammalian telomerase is a novel member of WD repeats family." Cell **88**(6): 875-84.

Nugent, C. I., G. Bosco, et al. (1998). "Telomere maintenance is dependent on activities required for end repair of double-strand breaks." Curr Biol **8**(11): 657-60.

Olovnikov, A. M. (1973). "A theory of marginotomy." J Theor Biol **41**: 181-90.

Pennock, E., K. Buckley, et al. (2001). "Cdc13 delivers separate complexes to the telomere for end protection and replication." Cell **104**(3): 387-96.

Peterson, S. E., A. E. Stellwagen, et al. (2001). "The function of a stem-loop in telomerase RNA is linked to the DNA repair protein Ku." Nat Genet **27**(1): 64-7.

Pluta, A. F., B. P. Kaine, et al. (1982). "The terminal organization of macronuclear DNA in Oxytricha fallax." Nucleic Acids Res **10**(24): 8145-54.

Pontaen, J., Stein, W. D. & Shall, S. (1983). "A quantitative analysis of the aging of human glial cells in culture." J. Cell. Phys. **117**: 342-352.

Prescott, J. and E. H. Blackburn (1997). "Telomerase RNA mutations in Saccharomyces cerevisiae alter telomerase action and reveal nonprocessivity in vivo and in vitro." Genes Dev **11**(4): 528-40.

Rabl, C. (1885). "Uber Zelltheilung." Morpholog. Jahrbuch **10**: 214-330.

Ritchie, K. B. and T. D. Petes (2000). "The Mre11p/Rad50p/Xrs2p complex and the Tel1p function in a single pathway for telomere maintenance in yeast." Genetics **155**(1): 475-9.

Romero, D. P. and E. H. Blackburn (1991). "A conserved secondary structure for telomerase RNA." Cell **67**(2): 343-53.

Runge, K. W. and V. A. Zakian (1996). "TEL2, an essential gene required for telomere length regulation and telomere position effect in Saccharomyces cerevisiae." Mol Cell Biol **16**(6): 3094-105.

Shampay, J., J. W. Szostak, et al. (1984). "DNA sequences of telomeres maintained in yeast." Nature **310**(5973): 154-7.

Shippen-Lentz, D. and E. H. Blackburn (1990). "Functional evidence for an RNA template in telomerase." Science **247**(4942): 546-52.

Singer, M. S. and D. E. Gottschling (1994). "TLC1: template RNA component of Saccharomyces cerevisiae telomerase." Science **266**(5184): 404-9.

Sperger, J. M. and T. R. Cech (2001). "A stem-loop of Tetrahymena telomerase RNA distant from the template potentiates RNA folding and telomerase activity." Biochemistry **40**(24): 7005-16.

Szostak, J. W. and E. H. Blackburn (1982). "Cloning yeast telomeres on linear plasmid vectors." Cell **29**(1): 245-55.

ten Dam, E., A. van Belkum, et al. (1991). "A conserved pseudoknot in telomerase RNA." Nucleic Acids Res **19**(24): 6951.

Tsukamoto, Y., J. Kato, et al. (1997). "Silencing factors participate in DNA repair and recombination in Saccharomyces cerevisiae." Nature **388**(6645): 900-3.

Tsukamoto, Y., A. K. Taggart, et al. (2001). "The role of the Mre11-Rad50-Xrs2 complex in telomerase- mediated lengthening of Saccharomyces cerevisiae telomeres." Curr Biol **11**(17): 1328-35.

Tzfati, Y., T. B. Fulton, et al. (2000). "Template boundary in a yeast telomerase specified by RNA structure." Science **288**(5467): 863-7.

Watson, J. D. (1972). "Origin of Concatemeric T7 DNA." Nat. New. Biol. **239**: 197-201.

Watson, J. D. and F. H. Crick (1974). "Molecular structure of nucleic acids: a structure for deoxyribose nucleic acid. G.D. Watson and F.H.C. Crick. Published in Nature, number 4356 April 25, 1953." Nature **248**(451): 765.

Wellinger, R. J., A. J. Wolf, et al. (1993). "Saccharomyces telomeres acquire single-strand TG1-3 tails late in S phase." Cell **72**(1): 51-60.

Zhong, Z., L. Shiue, et al. (1992). "A mammalian factor that binds telomeric TTAGGG repeats in vitro." Mol Cell Biol **12**(11): 4834-43.

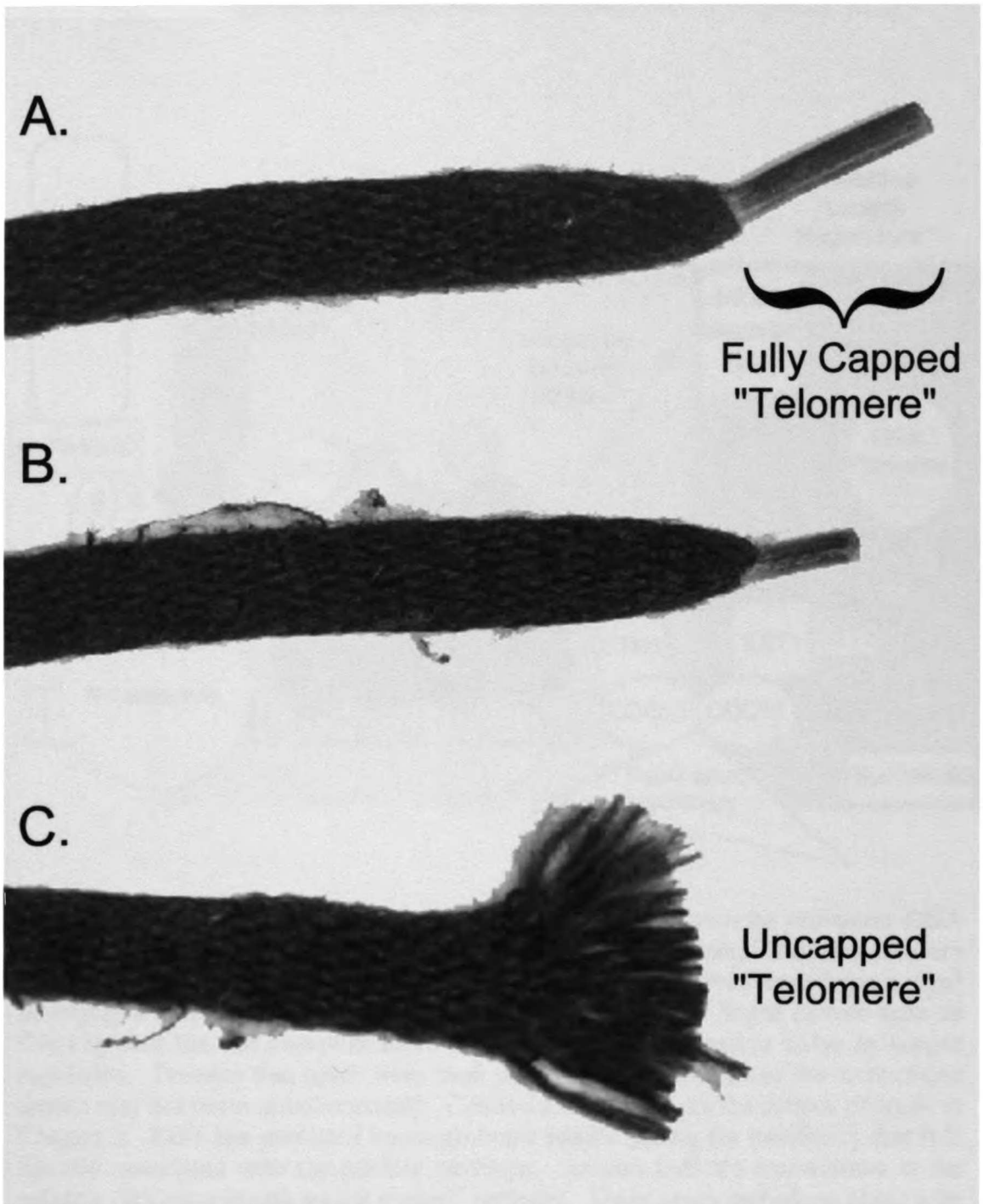


Figure 1 - The unravelling of this common shoelace represents the effects of telomere shortening on its ability to protect the chromosome end. The chromosome, represented by the shoelace, is protected at its end by the telomere, represented by the plastic cap, or aglet. A fully capped, functional chromosome is depicted in A. Progressive shortening of the "telomere" (B) eventually results in the uncapping of the "chromosome", seriously compromising its function.

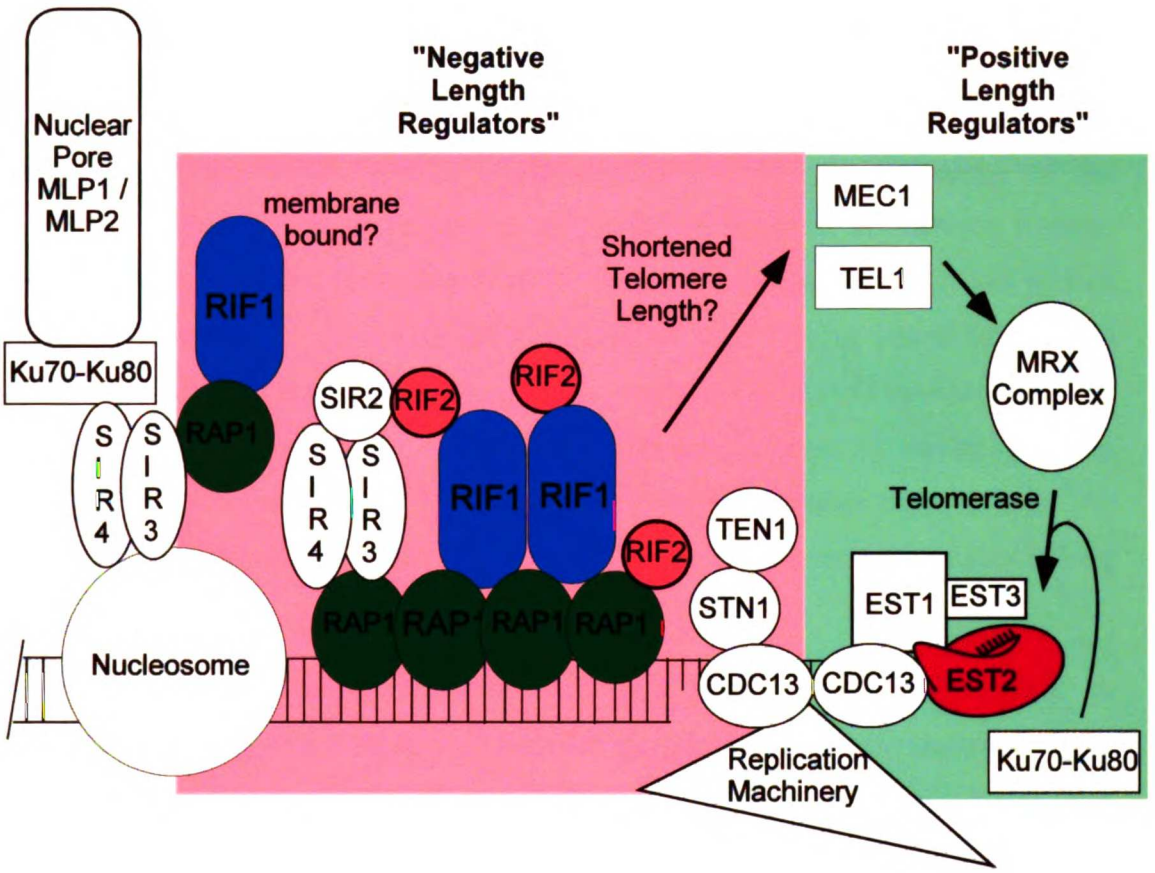


Figure 2 - This schematic represents the approximate position on telomeric DNA where some of the various proteins involved in telomere length maintenance are thought to act. Factors are roughly categorized by their positive (shaded green area) or negative (shaded pink area) role in telomere elongation. Some factors such as Cdc13p and the Ku complex have both positive and negative roles in length regulation. Proteins that touch have been shown to interact. All of the interactions shown may not occur simultaneously. Colored components are the subject of study in Chapter 3. RIF1 has predicted transmembrane motifs raising the possibility that it is directly associated with the nuclear envelope. Arrows indicate connections in the putative "telomere length sensor control" pathway. These genes and others along with a short description of their role in telomere biology are listed in Table 1.

Chapter 1 – Table 1 Legend

This table is a summary of protein coding genes that are directly and indirectly involved in telomere maintenance in *Saccharomyces cerevisiae*. This table is meant to be the basis for a searchable, sortable document to facilitate understanding of the relationships between the many complexes and activities involved in telomere biology. The headings shown in this table include the *S. cerevisiae* gene name and major protein modifications (denoted by *), the major homologs in other species (small letters) and number of species with high-ranking homologs as judged by BLAST scores to at least part of the protein. Also shown is a brief description of the protein's role in telomere-related processes and the effects of genetic ablation or over-expression of the protein. The last column indicates relevant protein families or complexes that the protein may belong to.

This table is certainly not complete and may be missing relevant annotations. A more complete version of this table, including the accession numbers for many homologs can be found in Appendix 4, the CD supplement. This table is partly compiled from information gathered by the Yeast Protein Database (Proteome Inc., **http://www.proteome.com**) and the BLink annotations (NCBI, **www.ncbi.nlm.nih.gov**) and the TelDB (http://www.genlink.wustl.edu/teldb/index.html). The completion of both the *Candida albicans* genome and a random-sequence-tag survey of 13 Hemiascomycetous yeast species offers the discovery of more homologs that may not be indicated in this table. Readers are encouraged to explore the Génolevures website (**http://cbi.labri.u-bordeaux.fr/Genolevures/my_geno.php3**) to find more yeast homologs to these telomere proteins. Abbreviations are shown below the table. Question marks indicate missing information. All data is the table is current as of January 1, 2002.

Chapter 1 – Table 1

scGene & *mod.	Homologs (h,m,d,ce, sp,sc,o)	sc Protein Function	Phenotype of ts or Null Mutation	Over-expression Phenotype	Family or Complex
ABF1 *p	d, ce, sc, km, kl 17 species	ARS-binding factor. Sites in STAR elements. Induced by MMS treatment. Essential.	?	?	?
ARD1	h, m, d, ce, sp, sc 27 species	Protein N-terminal acetyltransferase. Req'd for telomeric silencing. Interacts with Nat1p	Loss of TPE	?	Protein N-acetyltransferase
BDF2	h, m, d, ce, sp, sc 11 species	Bromodomain protein. Downregulated in senescing cells (S. Nautiyal). 5' UTR has PACE.	?	Slow growth	RSC family of DNA-ATPases Bromodomain
CDC13 *p	Similar folds to hPOT1, spPOT1 & ciliate *α*	Req'd for *in vivo* telomere maintenance. TG-rich single strand telomere binding, recruits telomerase via Est1p. Interacts with telomerase inhibitor STN1. Contains similar *α-β* fold as hPot1, spPot1, & ciliate *α-β* protein complex.	cdc13-est2 allele shows telomere shortening & senescence. cdc13-1 telomeres lengthen at non-permissive temp.	Telomere lengthening	Telomerase complex Perhaps same family as spPot1 and hPOT1
CTF18	h, m, d, ce, sp, sc 34 species	Involved in chromosome transmission fidelity & rDNA condensation.	Telomere shortening	?	RFC ATPases
DOT6	h, m, sc 27 species	Overproduction disrupts telomeric silencing but does not change telomere length. Putative MYB DNA-binding domain	No effect on TPE, HML, or rDNA silencing	Disrupts TPE	?
EST1	sc, kl 2 species	Required for *in vivo* telomere maintenance. Recruits Est2p via Cdc13p. FXDD RT motif, A,B,C,D RNA-dependent polymerase motifs. mRNA peaks in G1 phase.	Telomere shortening & senescence	?	Telomerase complex RNA binding protein
EST2	h, m, sp, c, kl, ca, xl, rn, a 14 species	Telomerase catalytic protein. Req'd for *in vivo* telomere maintenance.	Telomere shortening & senescence	Telomere lengthening Lifespan extension in mammlian systems	Telomerase complex Reverse Transciptase
EST3	?	Req'd for *in vivo* telomere maintenance. Function unknown. +1 programmed frameshift.	Telomere shortening & senescence	?	Telomerase complex
GAL11	d, ce, sp, sc, ca 21 species	RNA POL2 modulator. Modifies TPE	Shortened telomeres & reduced TPE	?	?
GBP2 / RLF6	h, m, d, ce, sp, sc, at, rn, xl 23 species	Single strand G-telomeric DNA binding protein. Mutation causes failure of Rap1p localization. 3 RNA recognition domains.	Rap1 delocalized but normal TPE	?	?
HDF1 / yKU70	h, m, sp, at, xl, gg 8 species	Interacts with HDF2-yKU80. Binds dsDNA ends. Req'd for TPE. Req'd for S-phase restriction of G overhangs. Req'd for NHEJ. Genetic Interaction with stem loop of *TLC1*	Loss of TPE at non-permissive temp.	Increased silencing at HML	?

scGene & *mod.	Homologs (h,m,d,ce, sp,sc,o)	sc Protein Function	Phenotype of ts or Null Mutation	Over-expression Phenotype	Family or Complex
HDF2 / yKU80	m, d, sp, xl, at, gg, tb 8 species	Interaction with HDF1-Ku70. Binds dsDNA ends. Req'd for NHEJ. Req'd for TPE. Req'd for S-phase restriction of G-strand overhangs.	Slightly shortened telomeres Loss of TPE	?	?
Histone 4 ***p,n,a**	Highly conserved in most species 70 species	Genes HHF1 and HHF2 code for histone H4 Interacts with SIR3 and SIR4	Mutation of N-terminus leads to decrease silencing & increased telomere length heterogeneity	Increased silencing Telomere Shortening	Histone
HPR1	at 7 species	Suppresses rap1-17 induced elongation & intrachromosomal excision recombination. Microtubule associated domain at C-terminus of mammalian homolog	Increased telomeric recombination	No effect on growth or recombination	THO Transcription Mediator Complex
HST1-4	h, m, d, ce, sp, sc, kl, ca 44 species	Similar to Sir2p. Likely histone deacetylase	?	?	NAD-dependent deacetylase
KEM1	h, m, d, ce, sp, sc, at 14 species	KAR(-) enhancing mutation protein. Indirect telomere effect through mRNA turnover. Has 5'-3' nuclease activity. Induced by DNA damage & meiosis	100 bp increase in telomere length	Slow growth	?
MEC1	h, m, d, ce, sp, sc, xl, at, gg 26 species	Kinase similar to human ATR. Central player in DNA damage response. Induced by UV irradiation, meiosis.	Telomere shortening. Synthetic senescence with TEL1	Telomere shortening Defective TPE	ATR Kinase PI3 Kinase DNA Damage Response
MEC3	?	G2 checkpoint. Involved in NMD2 regulation	100-fold decrease in TPE	?	Rad17-Mec3p-Ddc1p Complex
MLP1	h, m, d, ce, sp, sc, xl, at, gg 17 species	Myosin-like protein Req'd for telomere localization at nuclear periphery	mlp1, mlp2 double mutants have decreased TPE & delocalization of telomeres	Accumulation of poly A+ RNA in chromatin free area of nucleus	?
MLP2	h, m, d, ce, sp, sc, at 19 species	Myosin-like protein Req'd for perinuclear localization of yKU70 and localization of telomeres to nuclear periphery	mlp1, mlp2 double mutants have decreased TPE & delocalization of telomeres	?	Similar to Drosophila TPR proteins
MPT5 / UTH4	d,ce,sp,sc 17 species	Involved in telomere silencing and aging. Involved in mRNA degradation. Req'd for SIR3 localization to nucleolus in sir4 strains.	Stronger TPE & weaker rDNA silencing	Weakens TPE & strengthens rDNA silencing Extends lifespan	?
MRE11 *p	h,m, d,ce,sp, xl, at, gg 16 species	Req'd for *in vivo* telomere maintenance. Binds ds DNA. Induced by meiosis. Req'd for TRD. Putative phophoesterase.	Telomere shortening. Reduced levels of TRD.	?	MRX Complex DNA repair

scGene & *mod.	Homologs (h,m,d,ce, sp,sc,o)	sc Protein Function	Phenotype of ts or Null Mutation	Over-expression Phenotype	Family or Complex
NAT1	h, d, ce, sp, at 12 species	N-terminal acetyltransferase catalytic subunit. Req'd for TPE.	Loss of TPE HU sensitivity	Chromosome instability HU sensitivity	Protein N-acetyltransferase
NDJ1	?	Localizes at telomeres. Induced by meiosis. Responsible for meiotic telomere clustering.	Loss of meiotic telomere clustering	Interferes with meiotic chromosome segregation	?
NMD2	h, d, ce, sp, sc, at 25 species	RNA turnover regulation for many telomeric genes including EST1-3, SAS2, STN1, and ACT1	?	?	Nonsense-Mediated mRNA decay Complex Recruitment complex
NSR1 ***p**	h, m, d, ce, sp, sc 33 species	Nucleolin homolog. Binds strongly to ss telomeric DNA. Nuclear protein with 2 RRM domains involved in 20S to 18S rRNA processing. Induced by cold, repressed by MMS & S-phase	No effect on length or TPE	Inviability	RRM domains GAR Family
PIF1	d, ce, sp, sc 27 species	ssDNA-dependent ATPase req'd for mtDNA function Nuclear version inhibits telomerase.	Increased *de novo* telomere formation. Telomeres lengthen. No effect on TPE & chrom. loss	Telomere shortening Slow growth	Helicase
POL1	h, m, d, ce, sp, sc 99 species	DNA polymerase alpha, Inhibitor of telomerase. Induced by MBP1, SWI6, DNA damage	pol1-17 has considerable G-strand elongation. No TPE & reduced rDNA silencing	No effect on growth	DNA polymerase Alpha-Primase complex
PUF4 / YGL023	h, m, d, ce, sp, sc 17 species	Involved in silencing & aging. Required for SIR3-4 relocalization to nucleolus in sir4-42. Synthetic lethal with mpt5. Similar to mpt5	No effect on lifespan	?	PUF family of Pumilo repeat proteins
RAD50	h ,m, d, ce, sp, sc 18 species	Req'd for NHEJ, TRD, ds DNA break repair, telomere length maintenance. Myosin homology (like RIF1).	Req'd for TRD, telomere shortening, type II survivors	?	MRX Complex
RAD51	h, m, d, ce, sp, sc 97 species	Promotes strand exchange between homologous ss and dsDNA. Req'd for Type I survivor formation	Type I survivor formation impaired	Overproduction suppresses DNA damage and MMS sensitivity	Recombinosome Complex. Similar to E. coli RecA
RAD52	h, m, sp, sc, kl 9 species	DNA repair and recombination protein. Required for most survivor formation. Induced by UV and X irradiation, Meiosis	Some delocalization of Sir3p	No effect on telomere length or TPE	?
RAD6	h, m, d, ce, sp, sc 45 species	Ubiquitin-conjugating protein for monoubiquitination. Meiosis trx peak. Induced by DNA damage, UV irradiation	Reduced TPE	?	Ub processing
RAD9 ***p**	h, m, ce, sp, sc 32 species	DNA response & repair protein. Req'd for yKU80, Sir3p, Sir4p relocalization after DNA Damage	Telomere shortening	Oversuproduction suppresses yKu80	DNA damage reponse

scGene & *mod.	Homologs (h,m,d,ce,sp, sc,o)	sc Protein Function	Phenotype of ts or Null Mutation	Over-expression Phenotype	Family or Complex
RAP1 *p	h, sp, kl 13 species	Major telomere dsDNA binding protein. Trx regulation of carbohydrate metabolism and ribosome synthesis. Induced by Reb1p. Tethers non-nucleosomal chromatin at HML, HMR, telomeres. Req'd for TPE.	rap1-17 results in telomere lengthening	Slow growth Chromosome loss	Repressive, telomeric chromatin. MYB DNA binding proteins
RCK1 *a, p	h, m, d, ce, sp, sc 34 species	Serine-Threonine Protein Kinase	?	overexpression increases telomere length	?
RFC1	h, m, d, ce, sp, sc 89 species	Replication factor C	Telomere elongation. Reduced rDNA silencing	?	Replication Factor C Complex
RIF1	ca, sp 31 species mostly C-term.	Rap1 Interacting Factor. Rif2 interaction. Putative transmembrane domains. Myosin-like homology.	Telomere elongation, loss of rDNA silencing, shorter lifespan	Telomere shortening	Repressive Telomeric Chromatin
RIF2 **	?	Rap1 Interacting Factor. Rif1 interaction. Inhibits telomerase and Type II survivor formation. Lysine rich N terminus & basic C-terminus.	Telomere elongation Increased TPE	Telomere shortening	Repressive Telomeric Chromatin
RLF2	h, m, d, sp, sc 16 species	RAP1 localization factor, subunit of CAF-I. Req'd for stable inheritance of silent chromatin.	No effect on telomere length regulation. Decreased TPE	No effect on telomere length regulation	Chromatin Assembly Complex
RPD3	h, m, d, ce, sp, sc 55 species	Affects transcriptional silencing up to 20 kB from chromosome end. Req'd for sporulation.	Increased TPE up to 20kb from end	?	Histone deacetylase
SAS10	h, sc, sp, at, d, ce 26 species	Involved in telomere silencing	G2/M arrest	Derepresses HML, HMR, rDNA and telomeres	?
SAS2	h, m, d, ce, sp, sc 13 species	Req'd for establishment of silencing in absence of Sir1p. Putative acetyltransferase activity	Loss of TPE and HML/R silencing	?	SAS complex
SDS3	h, m mostly N-term. 4 species	Involved in telomere silencing. Req'd for RPD3-Sin3 histone deacetylase complex	Null has greater TPE	?	MYST subfamily, histone deacetylase
SET1	h, m, d, ce, sp, sc mostly in C-term. 25 species	Involved in telomere silencing. Interaction with MEC3. Part of protein methylation complex.	Telomeres about 50 bp shorter	Suppresses set2-1 silencing defect	Trithorax Family SET domain family
SGS1 *p	h, m, d, ce, sp, sc 70 species	Helicase, involved in aging. Werner's Syndrome homolog. Req'd for Type II survivor formation.	No Type II survivors in absence of Est2p	?	DEAD, DEAH Helicase Family
SIN3 *g	h, m, d, ce, sp 12 species	Transcriptional repression and activation. Induced by Reb1p	?	?	Histone deacetylase B SIN3 complex
SIR1	?	Silent information regulator. Mating type specific.	Loss of HML, HMR silencing	?	

scGene & *mod.	Homologs (h,m,d,ce,sp, sc,o)	sc Protein Function	Phenotype of ts or Null Mutation	Over-expression Phenotype	Family or Complex
SIR2	h, m, d, ce, sp, sc 45 species	Silent information regulator at HML, telomeres & rDNA. Histone deacetylase activity req'd for TPE. Involved in aging. Req'd for Sir3p localization in sir4 strain. Req'd for NHEJ.	TPE disrupted	?	NAD-dependent deacetylase RENT complex
SIR3 ***p**	m, sp, sc, kl Similar to Orc1 16 species	Req'd for TPE. DNA damage response. Tethered to telomeric chromatin by Rap1p. Req'd for peripheral localization of telomeres	Slightly shorter telomeres TPE disrupted	Spreading of silent chromatin in from the telomeres	AAA+ family of putative ATPase
SIR4 ***p**	h, d, ce, sp, kl, sc 40 species	Required for TPE. DNA damage response. Tethered to telomeric chromatin by Rap1p. Req'd for peripheral localization of telomeres. Inhibits rDNA silencing of Sir2p. Function in NHEJ.	Slightly shorter telomeres TPE disrupted	?	?
SPT2	ce 11 species	Suppressor of Ty element recombination	Increased Ty element recombination	?	HMG-like
STM1	Sp 17 species	Guanine-rich DNA or RNA quadraduplex binding protein. Binds triplex DNA. Very high abundance. Almost no affinity for ss or ds DNA.	ts growth on sorbitol or glycerol	?	?
STN1	?	Inhibits telomerase Recruited by Cdc13p	S or G2 arrest. Stn1-101,-138,-157 cause increased telomere length	?	?
SUM1	d, sp, sc 34 species	Suppressor of sir3. SUM1-1 dominant allele increases telomeric silencing. Restores HML/MR silencing in rap1-17	?	?	?
TBF1	sp, dictyostelium 3 species	Subtelomeric STAR binding sites. Inhibits silent chromatin spreading. Essential gene.	?	Inviable	MYB family DNA binding protein like TRF1, TRF2
TEL1	h, m, d, ce, sp, sc 26 species	ATM-like kinase, that is upstream of MRX complex	Telomere shortening. Synergistic with mec1.	?	PI3 Kinase
TEL2	sp, at 3 species	Recognizes single strand and duplex telomeric DNA. S phase expression peak, sporulation peak.	Decreased TPE and shortened telomeres at non-permissive temperature	?	?
TEN1	?	STN1 interacting protein, important for length regulation. Accumulates ssDNA at non-permissive temperature.	Activates RAD9-dependent ssDNA accumulation checkpoint at non-permissive temp.	Suppresses stn1 inviability	Telomere Length Regulation

scGene & *mod.	Homologs (h,m,d,ce,sp, sc,o)	sc Protein Function	Phenotype of ts or Null Mutation	Over-expression Phenotype	Family or Complex
XRS2 *p	d, at, c 5 species	Required for telomere addition *in vivo*. DNA repair protein. Involved in NHEJ. Required for recruitment of telomerase or processing of it substrate.	Shortened telomeres Inability to add *de novo* sequence	?	MRX Complex
YGR280 *m	h, m, d, ce, sp, sc 21 species	PINX-like protein. PIN2 interacting protein. Putative telomerase inhibitor	?	?	?
Y'-Help1	m, d, ce, sp, sc 8 species	Normally repressed, M & G1 trx. Peak. May be ALT-mechanism recombination protein. Y' element encoded helicase. Contain 11 tandem repeats of SXTXATTTXSXX	?	?	?

List of Abbreviations

General

chrom. = Chromosome

ss = Single-stranded

ds = Double stranded

*mod. = protein modification

Req'd = Required

temp = temperature

Protein Family or Complexes

GAR = Glycine/Arginine Repeats

MRX = Mre11p-Rad50p-Xrs2p

NHEJ = Non-homologous end joining

PACE = Proteosome-Associated Control Element

RENT = Regulator of Nucleolar silencing & Telophase exit

RRM = RNA Recognition Motif

STAR = Sub Telomeric Antisilencing Region

TPE = Telomere Position Effect

TRD = Telomere Rapid Deletion

Trx = Transcription

UTR = UnTranslated region

Species

h = *Homo sapiens*

m = Mus musculus

d = *Drosophila melanogaster*

ca = *Candida albicans*

ce = *C. elegans*

sp = *S. pombe*

sc = *Saccharomyces cerevisiae*

c = ciliates

kl = *Kluyveromyces lactis*

tb = *Trypanosoma brucei*

gg = *Gallus gallus*

km = *Kluyveromyces marxianus*

at = *Arabidopsis thaliana*

o = other

Protein Modifications

*p = phosphorylated

*m = myristolated

*g = glycosylated

*a = acetylated

** = Rif2 modification, see chapter 3

Chapter 2

Uncapping and deregulation of telomeres lead to detrimental cellular consequences in yeast

The end crowns all,
And that old common arbitrator, Time,
Will one day end it.

William Shakespeare (1564 - 1616), "Troilus and Cressida", Act 4 scene 5

Abstract

Telomeres are the protein-nucleic acid structures at the ends of eukaryote chromosomes. Tandem repeats of telomeric DNA are templated by the RNA component (*TER1*) of the ribonucleoprotein telomerase. These repeats are bound by telomere binding proteins, which are thought to interact with other factors to create a higher-order cap complex that stabilizes the chromosome end. In the budding yeast *Kluyveromyces lactis*, the incorporation of certain mutant DNA sequences into telomeres leads to uncapping of telomeres, manifested by dramatic telomere elongation and increased length heterogeneity (telomere deregulation). Here we show that telomere deregulation leads to enlarged, misshapen "monster" cells with increased DNA content and apparent defects in cell division. However, such deregulated telomeres became stabilized at their elongated lengths upon addition of only a few functionally wild-type telomeric repeats to their ends, after which the frequency of monster cells decreased to wild-type levels. These results provide evidence for the importance of the most terminal repeats at the telomere in maintaining the cap complex essential for normal telomere function. Analysis of uncapped and capped telomeres also show that it is the deregulation resulting from telomere uncapping, rather than excessive telomere length *per se*, that is associated with DNA aberrations and morphological defects.

Introduction

Telomeres function to protect chromosomes from incomplete replication (Olovnikov, 1973, Watson, 1972), end-to-end fusions (McClintock, 1941, van Steensel, et al., 1998), and loss (Kyrion, et al., 1992, Sandell and Zakian, 1993). The G-rich strand of telomeric DNA is templated and synthesized by the cellular ribonucleoprotein telomerase (Greider, 1995). Telomeric DNA is bound by proteins, forming a chromosome-protective cap (reviewed in (Grunstein, 1997)). It was shown previously that mutation of the *Tetrahymena thermophila* telomerase RNA gene (*TER*) results in the addition of mutant telomeric DNA to the chromosome termini, and dramatic cellular phenotypes (Lee, et al., 1993, Romero and Blackburn, 1995, Yu, et al., 1990). These include blockage of cells in late anaphase with failed chromosomal separation and somatic nuclei containing much greater than normal DNA content (Lee, et al., 1993, Yu, et al., 1990), suggesting that proper telomere function is important for completing nuclear division and mitosis (Kirk, et al., 1997). Cells lacking either the *TER* gene or telomerase activity experience progressive telomere shortening with each cell division (Bodnar, et al., 1998, Counter, et al., 1992, Lundblad and Szostak, 1989, McEachern and Blackburn, 1995, Singer and Gottschling, 1994) until telomeric DNA is reduced below a critical length, resulting in chromosome instability and failure of cells to proliferate. These results highlight the requirement for both the presence of telomeres and a minimal telomere length in order to form a functional telomeric cap complex.

In most species, including budding yeasts, telomere length is normally maintained within a narrow size range that is species-specific (Henderson and Petes,

1992, Walmsley and Petes, 1985). Although the telomeric "set length" within a yeast strain may be affected by temperature (McEachern and Hicks, 1993), carbon-source, or growth conditions (E.H.B., unpublished results), length regulation is a robust process that keeps the length of a given telomere within a relatively homogenous, tightly regulated range. In the budding yeasts *Saccharomyces cerevisiae* and *K. lactis*, telomere length has been experimentally manipulated by mutation of either telomeric DNA itself (via mutagenesis of the telomerase RNA gene template sequence), the telomerase enzyme, or the protein factors associated with the telomere (Prescott and Blackburn, 1997) (Roy, et al., 1998). In budding yeasts, the repressor-activator-protein (Rap1p) binds duplex telomeric DNA repeats (Berman, et al., 1986, Buchman, et al., 1988). Alleles of *RAP1* with alterations in its C-terminal domain (*rap1^t*) or its interacting factors *RIF1 and RIF2* can have large effects on telomere set length and the ability of cells to tightly regulate telomere size at any length, resulting in increased length heterogeneity in the telomere population (Hardy, et al., 1992, Kyrion, et al., 1992, Wotton and Shore, 1997). Coincident with these telomeric alterations, *rap1^t* strains can experience a 20-30-fold increase in the frequency of chromosome loss (Kyrion, et al., 1992). Mutations in analogous Myb-like DNA binding proteins that interact with telomeric DNA in other species also affect telomere set length and heterogeneity, as well as chromosome stability (Broccoli, et al., 1997, Cooper, et al., 1997, van Steensel, et al., 1998).

We chose to study the effects of telomere uncapping in *K. lactis*, because of its advantages as a model system for studying telomere-related processes. Unlike *S. cerevisiae*, which has irregular repeats (Shampay, et al., 1984), the *K. lactis* telomeric repeat is a 25 bp sequence that is copied precisely into telomeres (McEachern and

Blackburn, 1994, McEachern and Blackburn, 1995). This allows for the reliable incorporation of restriction sites into the telomere and hence the ability to follow such individual, marked mutant repeats in telomeres over many generations. In addition, *K. lactis* lacks the chromosome-internal telomeric repeat tracts that, in *S. cerevisiae*, can recombine with telomeres (Lundblad and Blackburn, 1993, Walmsley, et al., 1984), making it problematic to track the origin of telomeric repeats appearing at the chromosome ends. However, eleven of *K. lactis'* twelve telomeres share sub-telomeric homology and can recombine with one another, giving mutations incorporated into one telomere the potential to spread into the majority of the chromosome ends. In *K. lactis*, mutating the telomeric DNA, both within and outside of the Rap1p binding consensus sequence (Fig. 1 a), can result in two distinct types of changes at telomeres: the telomeres quickly elongate and show greatly increased length heterogeneity (deregulation). Such telomere deregulation has been attributed to disruption of the cap complex and consequent loss of end protection (Krauskopf and Blackburn, 1996, Krauskopf and Blackburn, 1998). The *ter1-Acc* mutation in the *K. lactis* telomerase RNA template disrupts Rap1p binding *in vitro* and results in immediate, dramatic telomere lengthening within 50 generations of introduction of the *ter1-Acc* gene (McEachern and Blackburn, 1995). These telomeres are also highly deregulated, as judged by their massive length heterogeneity in Southern blot analyses, manifested as a smear of fragments ranging in size from smaller than wild-type (250-500 bp) to many kilobases (≥25 kb). This degree of lengthening implies that over 500 bp of telomeric DNA was added per cell division in the *ter1-Acc* strain, compared to the normal average of 5 bp required to maintain stable telomeric DNA length in wild-type yeasts

(McEachern and Blackburn, 1995, Singer and Gottschling, 1994). Two other mutations, *ter1-Bgl* and *ter1-Kpn*, cause the telomeres initially to remain short and regulated for many generations, and then to abruptly elongate and deregulate much like *ter1-Acc* (McEachern and Blackburn, 1995). This telomere deregulation and elongation occurs only many generations (>750) after the introduction of the mutant *ter1* gene. Interestingly, the *ter1-Bgl* and *ter1-Kpn* mutations are located outside the telomeric Rap1p consensus binding site, and do not affect *in vitro* Rap1p binding affinity (Krauskopf and Blackburn, 1996).

Previous studies of the *ter1-Acc*, *ter1-Bgl*, and *ter1-Kpn K. lactis* mutants have focused on their kinetics of telomere uncapping and elongation as well as their effects on telomere cap-prevented recombination (McEachern and Blackburn, 1994, McEachern and Blackburn, 1995). Here, using these same mutants, we analyze how the telomere deregulation and elongation resulting from telomere uncapping affects both cell populations and individual cells. In addition, we specifically addressed whether it is the mean length of telomeres, or the cell's ability to regulate telomere length, that is important for cell viability. Previous results have provided evidence that the distal repeats of the telomere are especially important in end protection. *K. lactis* strains containing either the *ter1-AA, ter1-Bsi, te1-Kpn* template mutant genes in combination with a C-terminally deleted *RAP1* allele experience rapid telomere elongation and colony inviability like that in the Acc mutant (Krauskopf and Blackburn, 1996, Krauskopf and Blackburn, 1998). Addition of wild-type repeats to the *ter1-AA* mutant telomeric termini (re-capping) converts the telomeric DNA from an unregulated smear to a regulated pattern of discrete bands when visualized by Southern blot analysis. We

similarly re-capped *Acc, Bgl,* and *Kpn* strains with a phenotypically silent marked telomeric repeat to investigate how a WT-like distal end affects these mutant telomeres and the cellular phenotypes.

We report here that the deleterious effects of the *ter1-Acc*, *ter1-Bgl*, and *ter1-Kpn* mutations are distinct from those in senescing cells lacking the *TER1* gene, and are completely reversed by the addition of a functionally wild-type cap to the terminal mutant telomeric repeats. We conclude that the observed cellular phenotypes of these mutant strains are caused by the telomere deregulation resulting from cap complex disruption and subsequent loss of end protection, rather than extreme telomere elongation.

Materials & Methods

Strains

All *K. lactis* haploid strains were derived from the parental haploid strain K7B520 that has been previously described (McEachern and Blackburn, 1995). K7B520 was transformed with up to 10-50 ng/μl of pTER-BX plasmid, a YIP5 derivative containing the wild-type *TER1* gene with described template mutations (Fig. 1 a). Cells were electroporated (1.5kV, 200Ω, 25μF) and plated on medium lacking uracil and containing 1M sorbitol. After two days, transformants were re-streaked onto 5-fluoroorotic acid-containing medium and resistant colonies were screened for the desired gene replacement by digestion of PCR products with the restriction enzyme whose site was formed in the mutated template. PCR primers used were: upper strand, 5' GTC AAG TTC TGG AGG & lower strand, 5'CGA AGA GAA GAA TGG (Gibco-BRL, Gaithersburg MD). Cells were passaged by re-streaking representative colonies onto YPD and grown for 3 days at 30°C.

Southern Blotting Analyses

Genomic DNA was prepared from cells grown in YPD at 30°C until late log phase. At least three independent isolates were cultured for each of the uncapped *ter1-Acc, ter1-Bgl,* and *ter1-Kpn* strains. At least three independent loop-outs were cultured for *ter1-Bcl* re-capped and *Δter1* strains. DNA was cut with *EcoR1* (New England Biolabs, Beverly MA) and the appropriate second restriction enzyme at 37°C overnight (47°C for *BclI*) and electrophoresed 0.8% agarose, 1x TBE gels at 40V for 24 hours or

1% 0.5x TBE pulsed-field-gels at 230V for 16 hours (50 msec pulse time). After depurination, samples were transferred to Hybond N^+ nitrocellulose (Amersham, Piscataway NJ) and cross-linked with 1200 μjoules using a Stratalinker 1800 (Stratagene, La Jolla CA). Hybridizations were carried out in 0.5M Na_2HPO_4, 7% SDS, 0.5mM EDTA at 50°C for 4 - 16 hours and washed twice for 5 minutes at 40-50°C with 200mM NaCl wash buffer. The WT telomeric sequence probe used for all blots shown was 5' TAA TCA AAT CCG TAC ACC ACA TAC C. Blots were exposed to autoradiography film (Kodak, Rochester NY) and scanned at 300 dots-per-inch resolution.

FACS Analysis

Triplicate *K. lactis* cultures were grown to an A_{600} of 0.5-1. Triplicate cultures from a single stock were used for each uncapped strain, while 3 independent loop-out strains were used for re-capped and *Δter1* strains. Cells were centrifuged at ~5000 rpm in a clinical centrifuge and washed twice in 1 ml of PBS. Washed cells were then fixed in 70% ethanol in PBS and diluted to 1 A_{600} Unit/ml (~2 x 10^7 cells/ml). A 1 ml sample of fixed cells was washed twice with 1 ml of PBS. Cells were resuspended in 500 μl of PBS + 1mg/ml RNase A (Qiagen, Valencia CA) and incubated overnight on a rotating platform at 4°C. Afterwards, 20 μg of Proteinase K (Boehringer-Mannheim, Indianapolis IL) was added and samples were incubated at 55°C for 1 hour. Cells were centrifuged at 5000 rpm in a microfuge, washed once with 1 ml of PBS and resuspended in 500 μl of 50 μg/μl propidium iodide (Sigma, St. Louis MO) for one hour at room

temperature in the dark. Stained samples were diluted 1:10 in PBS and 30,000 ungated events were counted at 650 nm wavelength on a Becton Dickinson FacsCalibur.

For statistical analyses, the mean of the 2N peak was measured and the >2.5N cutoff was calculated using gated plots for each sample. We arbitrarily chose "greater than diploid content" as exceeding 2.5N. Statistical t-tests were performed using Microsoft Excel 98. Gated histograms were imported into Adobe Photoshop, where line thickness and gray shade were manipulated for overlays in Figure 3.

Microscopy

The same fixed samples used for FACS analyses (see above) were used for microscopic analyses carried out in parallel. About 1 ml of 70% ethanol/PBS-fixed cells was centrifuged at 5000 rpm in a microfuge and washed twice with 500 µl of PBS. Cells were stained for 5-15 minutes at room temperature with 1 µg/ml 2,6 – diamidinophenylindole (DAPI) and washed twice with 1 ml of PBS. At least three isolates of each strain were resuspended in 500 µl of PBS and sonicated for 10-30 seconds at 30% duty with a Branson sonifier. Microscopy was performed using 2 µl of cells on a Nikon Microphot FXA microscope at 100x magnification and photographed onto Kodak 400 ISO film. The *Δter1* image for Figure 4 b was acquired on a Leica DMLB microscope with a 300 dots-per-inch color CCD camera. The total number of cells counted for each mutant is noted in Table 1. Photographs were scanned at 300 dots-per-inch resolution and then cropped in Adobe Photoshop.

Results

DNA analyses of strains containing uncapped telomeres

The telomeric DNA phenotypes of *ter1-Acc*, *ter1-Bgl*, and *ter1-Kpn* mutants have been reported previously (McEachern and Blackburn, 1995). For the present studies, these findings were confirmed using fresh cultures of the same strains and are summarized in Fig. 1 b. All three mutations resulted in dramatic telomere deregulation and elongation (Fig. 2a lanes, 3, 9, and 15 and Fig. 2 b, lanes 7, 13, and 20) compared to wild-type (Fig. 2 a, lane 1and Fig. 2 b, lane 1). We define deregulation as the smeary, heterogeneous population of telomeric DNA species identified on these Southern blots. The apparent sizes of the heterogeneous telomeres in these mutant strains ranged from less than the smallest wild-type telomeric restriction fragment to $\geq$25 kb. The elongated mutant DNA was largely made up of mutant telomeric DNA repeats, as shown by secondary digestion with each restriction enzyme whose site was copied into telomeric DNA by the mutant telomerase (Fig. 2 a, lanes 4, 10, and 16). After cleaving off the mutant repeats, the length of these secondarily digested telomeric fragments reflects the remaining length of the original WT repeat tract that is located internally to the added mutant repeats on the telomere (Fig. 2 c). These sizes ranges of these internal wild-type repeat tracts were generally similar in all the mutants studied (bracketed area in Fig. 2 a, lanes 1, 4, 7, 10, 13, and 16). While the range of telomere sizes in *ter1* template mutants were comparable to WT, the individual telomere lengths were slightly shorter than WT after the deregulation following uncapping (Fig. 2a compare lane 1 to 4, 10, and 16). In addition, the patterns were different from the WT

patterns. This change in the patterns of telomeres has been shown previously to be due to the extensive sub-telomeric recombination, and has been documented in *ter1*-Acc and late passage *Bgl* and *Kpn* strains (McEachern and Blackburn, 1995). The 3.5 kb telomeric fragment that lacks sub-telomeric homology to the other telomeres (cut by *AccI* in lanes 4 and 5) does not appear to participate in these recombination events (McEachern and Blackburn, 1996). Since the telomeres in these strains are more recombinogenic than WT, individual lines passaged by serial restreaking of single colonies have telomeres patterns that are distinct both from other clonal lineages and the same lineage analyzed at different timepoints (Fig. 2a, compare lanes 7 to 10, 13 to 16). In an extreme case, *ter1-Bgl* recombined all of its homologous sub-telomeres into one species (Fig. 2a, lane 10, lower band). The telomeres of strains that had their *TER1* genes deleted for ~50 generations (*Δter1*) were homogeneous and slightly shorter than WT (Fig. 2 b, lane 5). In the subset of *Δter1* cells that survived senescence, the telomere patterns were also significantly altered (Fig. 2b, lane 6). In summary, while the telomeres of *ter1* template mutants were mostly deregulated and elongated, those of *Δter1* survivors were quite short and formed discrete size classes that were regulated. Thus, these short *Δter1* telomeres were distinct from the degraded telomeric species observed in the three *ter1* template mutant strains.

We used FACS analysis to investigate the cellular DNA content of *ter1* template mutant and *Δter1* cell populations (Fig. 3). In the WT *K. lactis* control strain, 13% of the cells contained DNA in excess of diploid content (Fig. 3 a, black histogram). Similarly, 10-16% of early passage (i.e. ~150 generation) *ter1-Bgl* and *ter1-Kpn* cells,

which have short well-regulated telomeres (Fig. 2 b, lanes 10 and 17), had greater than diploid DNA content (data not shown). While pre-senescent *Δter1* cells had a DNA content profile similar to WT (data not shown), post-senescent *Δter1* survivor cultures with short, relatively homogeneous telomeres (Fig. 2 b, lane 6) exhibited a 27% sub-population of cells with greater than diploid DNA content (Fig. 3 a, dashed histogram). Likewise, *ter1-Acc* cells, and late passage (>750 gens.) *ter1-Bgl* and late *ter1-Kpn* cells, which all had deregulated telomeres, showed 27%, 35%, and 19% sub-populations of cells with greater than diploid DNA content respectively (Fig. 3 b and c, black histograms, and data not shown). While the *Acc* and late passage *Bgl* mutants were significantly different from WT (both $p<0.01$), the variability of the WT slightly decreased the significance of the difference from the late passage *Kpn* mutant ($p=0.06$). The increase in DNA content in the *ter1* template sequence mutants and *Δter1* survivor strains reproducibly coincided with a decreased percentage of cells with 1N DNA content (Fig. 3, compare 1N peak in black histograms to gray histograms in a, b, c). These DNA content changes are unlikely to be explained by an increase in telomeric DNA alone, since the *Δter1* survivors had much less telomeric signal than the *ter1* mutants but still exhibited an increased DNA content (Fig. 2 b, lane 6, Fig. 3 a, dashed histogram). Furthermore, even assuming that all 12 telomeres in haploid *K. lactis* lengthened to an average of 100 kilobases, this would only represent approximately one tenth of the haploid genome size (Seoighe and Wolfe, 1998). Hence, the increased DNA content of these *ter1* mutants was likely to have resulted from either endoreduplication and/or defects in chromosome segregation.

Microscopic analyses of *ter1* mutants

To determine whether DNA segregation was affected in *ter1* template sequence mutants, we used fluorescence light microscopy and DAPI-staining to examine the cellular DNA, and brightfield microscopy to examine overall cell morphology and cell budding indices (Figs. 4-6, Table 1). We predicted that if DNA segregation were affected, then multiple or large DAPI staining structures should be visible in a single cell body and some percentage of cells might contain little or no DNA. While WT and pre-senescent *Δter1* cells looked indistinguishable (Fig. 4 a and data not shown), post-senescent *Δter1* survivors had a 4% population of somewhat enlarged, misshapen cells with abnormally distributed DNA (Table 1). These cells also had very degraded cell walls and collapsed buds as judged by brightfield microscopy (Fig. 4 b, arrowheads).

We found that 10% of *ter1-Acc* mutant cells had cellular defects. These were distinctly different from, and more severe than, the most extreme morphological defects of post-senescent *Δter1* cells. Many *ter1-Acc* mutant cells had multiple DAPI-staining structures (Fig. 4 d and e, arrows), while others had no brightly staining DAPI structures but did contain large areas of diffuse DAPI staining (data not shown, see similar phenotypes for *Bgl* and *Kpn* mutants in Figs. 5 d and 6 d). These *Acc* cells often appeared to have budding and division defects. They were frequently grossly enlarged or elongated (Fig. 4 d and e) and some cells formed chains that were resistant to extensive sonication (Fig. 4 e). Other cells were spherical but enlarged to up to 5 times the diameter of wild type cells (Fig. 4 d). We use the term "monster cells" generally to

describe these phenotypes, with a given cell needing only to exhibit one of these traits to qualify as a monster cell.

The *ter1-Bgl* and *ter1-Kpn* mutations also resulted in monster cell phenotypes, but only in cell populations with deregulated, elongated telomeres. Thus, early passage (~150 generation) *ter1-Bgl* and *ter1-Kpn* strains with short, regulated telomeres showed no significant monster cell phenotypes above background levels (Figs. 5 a and 6 a), while isogenic isolates passaged for >750 generations, and with deregulated telomeres, exhibited high levels of severe monster cell phenotypes. The percentages of monster cells in the populations of late passage *ter1-Bgl* and *ter1-Kpn* cells strains were 12% and 13% respectively (Table 1). The same monster cell populations also contained either multiple DAPI-staining structures (Fig. 5 c and 6 c & d, arrows) or decondensed DAPI-staining material (Figs. 5d and 6d, arrowheads) and apparent budding and division defects similar to the *ter1-Acc* mutant cells (Figs.4e, 5c, and 6c).

Our microscopic analyses highlight the differences between the abnormal phenotypes associated with senescence and monster cells, respectively. Although post-senescent *Δter1* survivors were phenotypically abnormal, they had irregular, degraded cell walls and collapsed buds unlike those of the *ter1* template sequence mutants. Furthermore, post-senescent *Δter1* survivors did not exhibit multiple DAPI staining structures within one cell. Finally, the incidence of monster cells in post-senescent *Δter1* populations was at most a third that of other *ter1* strains and was considerably more variable between lineages (Table 1). In summary, we concluded that telomere uncapping was caused by the *Acc, Bgl, or Kpn* mutations and resulted in telomere

deregulation and elongation. This correlated with a sub-population of cells containing DNA in excess of diploid amounts and a significantly increased percentage of morphologically aberrant monster cells that were distinct from post-senescent *Δter1* survivors. The multiple DAPI staining structures in all three uncapped *ter1* template mutant strains suggested that the cell's ability to segregate DNA was inversely correlated with the extent of telomere deregulation/elongation.

Telomere re-capping restores length regulation

We wished to dissect which property of the uncapped telomeres caused the extreme monster phenotypes described above: deregulation or extreme length. To address this issue we replaced the mutant *ter1* gene in *Acc, Bgl,* and *Kpn* strains with a *ter1-Bcl* allele, which adds phenotypically silent, functionally wild-type, repeats to the telomeric DNA end (M.J. McEachern and E.H. Blackburn, unpublished results; (Krauskopf and Blackburn, 1998, Roy, et al., 1998). The *Bcl* repeats contain a *BclI* restriction enzyme site, so that these added marked repeats can be distinguished from pre-existing wild type or other mutant repeats. The *Bcl* repeats bind Rap1p normally *in vitro* and thus were predicted to allow the previously disrupted telomere cap to reform at the distal end of the telomere. In all three mutant *ter1* strains studied, re-capping with *Bcl* repeats caused a transition from an deregulated smear of telomeric DNA to a series of discrete, length-regulated but still elongated telomeric bands (Fig. 2 b, arrows, compare lanes 7 to 8, 13 to 14, and 20 to 21). This transition occurred within ~50 generations (the earliest time point at which DNA could be analyzed). These re-regulated telomeres remained much longer than wild-type (Fig. 2 b, lanes 8, 14, and 21)

with a significant fraction of the telomere signal still at limit mobility (≥25 kb) for the *Bgl* and *Kpn* mutants (Fig. 2 b, lanes 14 and 21). Re-capping did not significantly change the sizes of the internal wild-type repeat tracts (Fig. 2 a, compare lanes 4 to 5, 7 to 8, 10 to 11, 13 to 14, 16 to 17). Digestion of the cap repeats with *BclI* revealed that only 3-4 *ter1-Bcl* repeats were added to each telomere (Fig. 2 b, compare lanes 8 to 9, 21 to 22). Interestingly, in late *ter1-Bgl* cells the *Bcl* repeats seemed to incorporate further into some late passage telomeres, since digestion of the cap resulted in large decreases in the sizes of some telomere restriction fragments (Fig. 2b, compare lane 14 to 15). The inward migration of these repeats may have been due to faster terminal repeat turn-over (Krauskopf and Blackburn, 1998) or recombination, since isogenic cells passaged for an additional 150 generations exhibited a significantly altered telomere pattern (Fig. 2b, compare lane 15 to 16).

To determine whether telomeric DNA shortened overall after re-capping with *Bcl* repeats, we performed quantitative analyses of the total telomeric hybridization signal in uncapped and re-capped lanes, for all three *tel* template mutants (Fig. 2 b and data not shown). We repeated these analyses using pulse-field gel electrophoresis and compared the total telomeric signals on four chromosomes between uncapped and re-capped strains (data not shown). In all cases, there was no significant decrease in telomeric signal following re-capping.

In summary, the internal WT repeat tracts of uncapped telomeres in *ter1* template mutants shortened only slightly, and were longer than those in post-senescent *Δter1* survivors. Re-capping added an average of 3-4 *ter1-Bcl* repeats to the distal tips of telomeres, although in some cases recombination events allowed migration of *Bcl*

repeats further into the telomere. In all cases, however, the re-capped *ter1* strains regained their ability to regulate telomere length about a new mean size, and the majority of telomeres remained significantly elongated.

Telomere re-capping restores normal cellular phenotypes in mutant *ter1* strains

The re-capped *ter1* template mutant strains were examined by FACS analysis (Fig. 3). Following re-capping, all three *ter1* strains eventually exhibited significantly fewer cells with greater than diploid DNA content. The percentage of re-capped *ter1-Acc* and *ter1-Bgl* cells with greater than diploid DNA content was the same (7%) as in re-capped wild-type cells (Fig. 3 a, b, and c, gray histograms). Interestingly, in *ter1-Bgl* strains, DNA content did not show an immediate large decrease upon re-capping (data not shown). However, ~150 generations after re-capping, the fraction of cells with greater than diploid DNA content was reduced to wild-type levels (Fig. 3 c, gray histogram). In contrast, re-capped late passage *ter1-Kpn* strains showed a significant decrease in cells with greater than diploid DNA content as soon as cells could be analyzed (from 19% to 9%; $p<0.001$, data not shown).

By the criteria of DAPI staining and brightfield microscopic analyses, the nuclear and cell morphologies of re-capped *ter1-Acc* and late passage *ter1-Kpn* strains were indistinguishable from wild-type (Figs. 4 f, 6 e, Table 1), even though their telomeres remained very long. The early passage re-capped *ter1-Bgl* and *ter1-Kpn* strains also had DNA contents and percentages of monster cells comparable to wild-type (data not shown, Figs. 5 b and 6 b, Table 1). Immediately after re-capping, the late passage *ter1-Bgl* strain still exhibited a 9% sub-population of monster cells (Table 1).

Qualitatively, these re-capped *ter1-Bgl* cells were not as large or grotesquely malformed as the uncapped *ter1-Bgl* monster cells (Fig. 5, compare c and d to e). However, ~150 generations after re-capping, the percentage of monster cells returned to wild-type levels (Fig. 5 f, Table 1) even though the telomeres in these cells appeared qualitatively similar to those immediately after re-capping (Fig. 2 b, compare lanes 15 to 16). Thus, while Southern blot analyses showed that *ter1-Bcl* repeats had been physically added to the distal ends of telomeres within 50 generations, it took additional time for late passage *ter1-Bgl* mutants to establish a cell population with functional telomeric caps.

Discussion

The DNA-protein complex at the end of telomeres is thought to be important for their chromosome-protective functions. When this distal cap complex is disrupted by adding mutant repeats or shortening the existing telomere beyond a critical length, the chromosome becomes uncapped and subject to damage. Uncapping can be defined as the loss of end protection and results in either net telomere shortening or lengthening, increased recombination in telomeric regions, and/or the loss of regulation about a mean telomere length. Here we have addressed two questions related to telomere length regulation in *K. lactis*: First, what are the cellular phenotypic consequences of uncapped telomeres in *ter1* template sequence mutants and post-senescent Δ*ter1* survivor strains? Second, upon finding that cells respond poorly to telomere uncapping, we asked whether it is the resulting telomere deregulation, as opposed to elongation *per se*, that is correlated with the observed phenotypes.

This is the first detailed report in yeast of the cellular morphological consequences caused by telomere uncapping. Telomere uncapping in *ter1* template sequence mutants was correlated with a greater than diploid DNA content, aberrant nuclear morphologies, and apparent cell division defects. We conclude that it is the deregulation of telomeres resulting form uncapping, rather than their elongation, that is associated with these phenotypes. The addition of a few wild-type-like repeats to the extreme terminus of the elongated mutant *ter1* telomeres allowed strains to regain their ability to regulate telomeres, even though the telomeres were up to 100 times longer than wild-type. Interestingly, the *ter1-Bgl* mutant telomeres were not fully capped at

first and *Bcl* repeats migrated further into the telomeres than in other mutants. This may have been due to continued degredative shortening of the telomeres followed by *de novo Bcl* addition or recombination of the *Bcl* cap with the internal tracts. However, after being re-capped for ~150 generations, *Bgl* mutant strains behaved similarly to the *Acc* and *Kpn* mutants. Hence, telomere re-capping eventually caused the DNA content and cellular morphology to return to normal in all three *ter1* mutants.

The mechanism by which the deregulation of uncapped telomeres leads to monster cell formation in *K. lactis* is not known. While general genomic instability and consequent misregulation of gene expression may result in monster cells, the addition of a wild-type telomeric cap is sufficient for recovery of the cell population. In *S. cerevisiae*, senescing cells show increased chromosome loss (Lundblad and Szostak, 1989). Likewise, elongated, poorly regulated telomeres can increase chromosome loss rates (Kyrion, et al., 1992). Telomere uncapping can lead to either telomere shortening (*Δter1*) or deregulation/elongation (*ter1* template mutants); we have shown here that each has distinct telomere and monster cell phenotypes. The*Δter1* survivors had cell walls that appeared degraded and they did not show multiple DAPI staining structures in one cell body. On the other hand, monster cells of *ter1* template sequence mutants had healthy-looking cell walls, decondensed chromatin, often up to 10 nucleus-sized DAPI-staining objects in a single cell body, with frequently no DNA in the adjacent body. Evidence supporting DNA segregation or replication defects includes the observation that the DNA content of cultures with elongated, uncapped telomeres was greatly increased. Taken together with the observation of cells with either increased DAPI

staining or no staining and the morphological results, these results strongly suggest that deregulated telomeres can cause DNA missegregation.

A model for the effects of deregulated, uncapped telomeres on chromosome segregation and cell morphology

We propose the following model for how uncapped telomeres may negatively affect cells (Fig. 7). While deletion of *ter1* results in telomere shortening until the cap is lost, addition of certain mutant repeats can disrupt the cap without telomere shortening. Mutant repeats that cannot bind Rap1p (i.e. *Acc*) result in immediate telomere uncapping, while mutant repeats that retain Rap1p binding (*Bgl* and *Kpn*) do not result in immediate uncapping. The effects of the *Bgl* and *Kpn* mutations accumulate over time (McEachern and Blackburn, 1995) until some as yet undefined change(s) in the properties of the Rap1p-nucleated complex on the mutant telomeric DNA prevents functional end protection. Uncapped telomeres may over-elongate by telomerase-mediated or recombination pathways at this point (Krauskopf and Blackburn, 1998, McEachern and Blackburn, 1996). Such telomeres are also subject to degradation, as shown by the smear of telomeric signal migrating faster than wild-type telomeres (Fig. 2 b, lanes 7, 13, and 20). Uncapped, elongated may be recognized as damage, causing cell cycle delay or accidental repair/telomeric fusion, resulting in dicentric chromosome formation. Individual chromosomes or whole genomes may be lost or mis-segregated. This genomic instability results in further negative phenotypic consequences for the cell. Once polyploidy or aneuploidy occurs, strong selection pressures exist for the healthiest cells, suggesting why the majority of cells in a population are not visually aberrant. However, microcolony analyses of phenotypically

wild-type *ter1-Acc* mutant cells revealed that they continually give rise to sub-populations of monster cells (data not shown).

Re-capping reverses the phenotypic effects of telomere deregulation. Re-establishment of a functional cap may occur immediately for the population, as in the cases of the re-capped *ter1-Acc* and *ter1-Kpn* strains, or be slower as in the case of the late passage *ter1-Bgl* mutant. We propose that re-capping involves reforming a stable protein-DNA complex at the telomere ends, preventing these chromosomes from becoming deregulated and exerting detrimental effects. Cells with stably capped telomeres are likely to have a substantial growth advantage, and once a *ter1* population is re-capped the frequency of unstable monster cells decreases as healthy cells take over the population.

The importance of the distal telomeric repeats for cap formation

The addition of 3-4 *ter1-Bcl* repeats to the termini of the telomere was sufficient to eventually cap *ter1-Acc*, *Bgl*, and *Kpn* mutant telomeres. The relatively few *Bcl* repeats that migrated into the *Bgl* telomeres did not appear to have a significant effect on the eventual capping of these telomeres. The telomeres in these re-capped strains contain three distinguishable, possibly functional domains: the remaining ~250-300 bp internal tract of original wild-type repeats most proximal to the centromere, the adjacent large tract of *Acc, Bgl,* or *Kpn* mutant repeats, which may exceed 25 kb in length, and the (usually) 3-4 functionally wild-type *ter1-Bcl* repeats at the very terminus of the telomere (Fig. 2 c). Whether the remaining internal wild-type repeats were necessary for the re-establishment of a normal cell population following re-capping is unknown.

However, notably, the total telomeric DNA hybridization signal in elongated *ter1* mutants remained unchanged after re-capping, providing evidence that re-capping is not obligatorily associated with a reduction in mean telomere length. This evidence strongly suggests that it is not telomere length, but the very terminal repeats that are important for preventing monster cell formation.

It is thought that functionally wild type telomeres assume a higher-order structure nucleated on Rap1p which protects the chromosome end. The C-terminus of Rap1p interacts with the Sir (Moretti, et al., 1994), and Rif 1 and 2 (Wotton and Shore, 1997) proteins. Generally, mutations that prevent Rap1p interaction with telomeric DNA (i.e. template mutations), Sirs and/or Rifs, or C-terminal mutations in Rap1p, result in telomere elongation, suggesting that these interactions help stabilize the telomeric complex that regulates telomere length (Krauskopf and Blackburn, 1996, Kyrion, et al., 1992). The results reported here also address whether the monster cell phenotypes observed are the pleiotropic effects of changing the amounts of Rap1p or associated factors in the cell. In the case of the *Acc* mutation, Rap1p binds with significantly lowered affinity *in vitro* (Krauskopf and Blackburn, 1996), and therefore Rap1p occupancy of these repeats *in vivo* may be low. Although 100-fold less Rap1p is predicted to bind *Acc* repeats, up to 100 times as many repeats may be present at each telomere in *ter1-Acc* strains. Therefore, the overall Rap1p levels at telomeres may not differ greatly between wild-type and *ter1-Acc* cells. Nevertheless, the structure of their telomeric complexes are likely distinct. In contrast, both *Bgl* and *Kpn* mutant repeats have normal Rap1p binding affinity *in vitro* and upon elongation could potentially titrate Rap1p, along with interacting proteins, away from the scores of genes they regulate.

Yet the functionally re-capped *ter1-Acc*, *ter1-Bgl* and *ter1-Kpn* strains all have as much telomeric DNA as uncapped strains and appear as healthy as wild-type. Therefore, it is unlikely that titration of Rap1p explains the phenotypes associated with monster cells.

It is possible that the uncapped *ter1* mutants are unable to regulate the single-stranded ends of the telomere and are therefore unable to regulate length. The *Acc*, *Bgl* and *Kpn* mutations may affect the interaction of putative end-binding factors, such *K. lactis* homologues of the Cdc13p, Est1p, or Stn1p proteins found in *S. cerevisiae* (Grandin, et al., 1997, Nugent, et al., 1996, Virta-Pearlman, et al., 1996). If these *ter1* mutant repeats were incapable of binding such end factors normally, this could expose the terminal region of the telomere to factors such as recombination-associated activities including degradation enzymes.

A functional cap complex at the telomere ends appears to be important in other organisms besides budding yeasts. Mutations in the mammalian telomere binding proteins TRF1 and TRF2 have been shown to result in varying degrees of telomere lengthening and chromosome fusions respectively (Bianchi, et al., 1997, van Steensel, et al., 1998). In *Schizosaccharomyces pombe* the telomere binding protein Taz1p has been shown to be important in telomere length control (Cooper, et al., 1997). Additionally, mutations in Taz1p that result in improper meiotic segregation, defects in telomere clustering, and low spore viability may reflect failure to form a functional cap (Cooper, et al., 1998, Nimmo, et al., 1998). Understanding the role of capping in telomere function will likely be useful in understanding the roles of telomeres in cell viability and division control.

Acknowledgments

We thank Mike McEachern for his generous gift of uncapped mutant *ter1* template sequence strains, the pTER-BX capping plasmid, and sharing unpublished results and Anat Krauskopf and Mike McEachern for many fruitful discussions. We also thank Simon Chan, Sandy Johnson, Andrew Murray, and Thea Tlsty for critical review of the manuscript, Andrew Murray's lab for use of their microscope, and Rudi Grosschedl's lab for use of their FacsCalibur. This work was funded by NIH grants GM26259 to E.H.B, a National Science Foundation Graduate Fellowship to C.D.S and NIH training grant T32CA09270.

References

1. Berman, J., C. Y. Tachibana, and B. K. Tye. 1986. Identification of a telomere-binding activity from yeast. *Proc Natl Acad Sci U S A.* 83:3713-7.

2. Bianchi, A., S. Smith, L. Chong, P. Elias, and T. de Lange. 1997. TRF1 is a dimer and bends telomeric DNA. *Embo J.* 16:1785-94.

3. Bodnar, A. G., M. Ouellette, M. Frolkis, S. E. Holt, C. P. Chiu, G. B. Morin, C. B. Harley, J. W. Shay, S. Lichtsteiner, and W. E. Wright. 1998. Extension of life-span by introduction of telomerase into normal human cells [see comments]. *Science.* 279:349-52.

4. Broccoli, D., A. Smogorzewska, L. Chong, and T. de Lange. 1997. Human telomeres contain two distinct Myb-related proteins, TRF1 and TRF2. *Nat Genet.* 17:231-5.

5. Buchman, A. R., W. J. Kimmerly, J. Rine, and R. D. Kornberg. 1988. Two DNA-binding factors recognize specific sequences at silencers, upstream activating sequences, autonomously replicating sequences, and telomeres in Saccharomyces cerevisiae. *Mol Cell Biol.* 8:210-25.

6. Cooper, J. P., E. R. Nimmo, R. C. Allshire, and T. R. Cech. 1997. Regulation of telomere length and function by a Myb-domain protein in fission yeast [see comments]. *Nature.* 385:744-7.

7. Cooper, J. P., Y. Watanabe, and P. Nurse. 1998. Fission yeast Taz1 protein is required for meiotic telomere clustering and recombination [see comments]. *Nature.* 392:828-31.

8. Counter, C. M., A. A. Avilion, C. E. LeFeuvre, N. G. Stewart, C. W. Greider, C. B. Harley, and S. Bacchetti. 1992. Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. *Embo J.* 11:1921-9.

9. Grandin, N., S. I. Reed, and M. Charbonneau. 1997. Stn1, a new Saccharomyces cerevisiae protein, is implicated in telomere size regulation in association with Cdc13. *Genes Dev.* 11:512-27.

10. Greider, C. W. 1995. Telomerase Biochemistry and Regulation. *In* Telomeres. E. H. Blackburn and C. W. Greider, editors. Cold Spring Harbor Laboratory Press. 35-68.

11. Grunstein, M. 1997. Molecular model for telomeric heterochromatin in yeast. *Curr Opin Cell Biol.* 9:383-7.

12. Hardy, C. F., L. Sussel, and D. Shore. 1992. A RAP1-interacting protein involved in transcriptional silencing and telomere length regulation. *Genes Dev.* 6:801-14.

13. Henderson, S. T., and T. D. Petes. 1992. Instability of simple sequence DNA in Saccharomyces cerevisiae. *Mol Cell Biol.* 12:2749-57.

14. Kirk, K. E., B. P. Harmon, I. K. Reichardt, J. W. Sedat, and E. H. Blackburn. 1997. Block in anaphase chromosome separation caused by a telomerase template mutation [see comments]. *Science.* 275:1478-81.

15. Krauskopf, A., and E. H. Blackburn. 1996. Control of telomere growth by interactions of RAP1 with the most distal telomeric repeats. *Nature.* 383:354-7.

16. Krauskopf, A., and E. H. Blackburn. 1998. Rap1 protein regulates telomere turnover in yeast. *Proc Natl Acad Sci U S A.* 95:12486-91.

17. Kyrion, G., K. A. Boakye, and A. J. Lustig. 1992. C-terminal truncation of RAP1 results in the deregulation of telomere size, stability, and function in Saccharomyces cerevisiae. *Mol Cell Biol.* 12:5159-73.

18. Lee, M. S., R. C. Gallagher, J. Bradley, and E. H. Blackburn. 1993. In vivo and in vitro studies of telomeres and telomerase. *Cold Spring Harb Symp Quant Biol.* 58:707-18.

19. Lundblad, V., and E. H. Blackburn. 1993. An alternative pathway for yeast telomere maintenance rescues est1- senescence. *Cell.* 73:347-60.

20. Lundblad, V., and J. W. Szostak. 1989. A mutant with a defect in telomere elongation leads to senescence in yeast. *Cell.* 57:633-43.

21. McClintock, B. 1941. The stability of broken ends of chromosomes in *Zea Mays*. *Genetics.* 26:234-82.

22. McEachern, M. J., and E. H. Blackburn. 1994. A conserved sequence motif within the exceptionally diverse telomeric sequences of budding yeasts. *Proc Natl Acad Sci U S A.* 91:3453-7.

23. McEachern, M. J., and E. H. Blackburn. 1995. Runaway telomere elongation caused by telomerase RNA gene mutations. *Nature.* 376:403-9.

24. McEachern, M. J., and E. H. Blackburn. 1996. Cap-prevented recombination between terminal telomeric repeat arrays (telomere CPR) maintains telomeres in Kluyveromyces lactis lacking telomerase. *Genes Dev.* 10:1822-34.

25. McEachern, M. J., and J. B. Hicks. 1993. Unusually large telomeric repeats in the yeast Candida albicans. *Mol Cell Biol.* 13:551-60.

26. Moretti, P., K. Freeman, L. Coodly, and D. Shore. 1994. Evidence that a complex of SIR proteins interacts with the silencer and telomere-binding protein RAP1. *Genes Dev.* 8:2257-69.

27. Nimmo, E. R., A. L. Pidoux, P. E. Perry, and R. C. Allshire. 1998. Defective meiosis in telomere-silencing mutants of Schizosaccharomyces pombe [see comments]. *Nature.* 392:825-8.

28. Nugent, C. I., T. R. Hughes, N. F. Lue, and V. Lundblad. 1996. Cdc13p: a single-strand telomeric DNA-binding protein with a dual role in yeast telomere maintenance. *Science.* 274:249-52.

29. Olovnikov, A. M. 1973. A theory of marginotomy. *J Theor Biol.* 41:181-90.

30. Prescott, J., and E. H. Blackburn. 1997. Telomerase RNA mutations in Saccharomyces cerevisiae alter telomerase action and reveal nonprocessivity in vivo and in vitro. *Genes Dev.* 11:528-40.

31. Romero, D. P., and E. H. Blackburn. 1995. Circular rDNA replicons persist in Tetrahymena thermophila transformants synthesizing GGGGTC telomeric repeats. *J Eukaryot Microbiol.* 42:32-43.

32. Roy, J., T. Boswell-Fulton, and E. H. Blackburn. 1998. Specific telomerase RNA residues distant from the template are essential for telomerase function. *Genes & Development.* in press.

33. Sandell, L. L., and V. A. Zakian. 1993. Loss of a yeast telomere: arrest, recovery, and chromosome loss. *Cell.* 75:729-39.

34. Seoighe, C., and K. H. Wolfe. 1998. Extent of genomic rearrangement after genome duplication in yeast. *Proc. Natl. Acad. Sci. USA.* 95:4447-44452.

35. Shampay, J., J. W. Szostak, and E. H. Blackburn. 1984. DNA sequences of telomeres maintained in yeast. *Nature.* 310:154-7.

36. Singer, M. S., and D. E. Gottschling. 1994. TLC1: template RNA component of Saccharomyces cerevisiae telomerase [see comments]. *Science.* 266:404-9.

37. van Steensel, B., A. Smogorzewska, and T. de Lange. 1998. TRF2 protects human telomeres from end-to-end fusions. *Cell.* 92:401-13.

38. Virta-Pearlman, V., D. K. Morris, and V. Lundblad. 1996. Est1 has the properties of a single-stranded telomere end-binding protein. *Genes Dev.* 10:3094-104.

39. Walmsley, R. M., and T. D. Petes. 1985. Genetic control of chromosome length in yeast. *Proc Natl Acad Sci U S A.* 82:506-10.

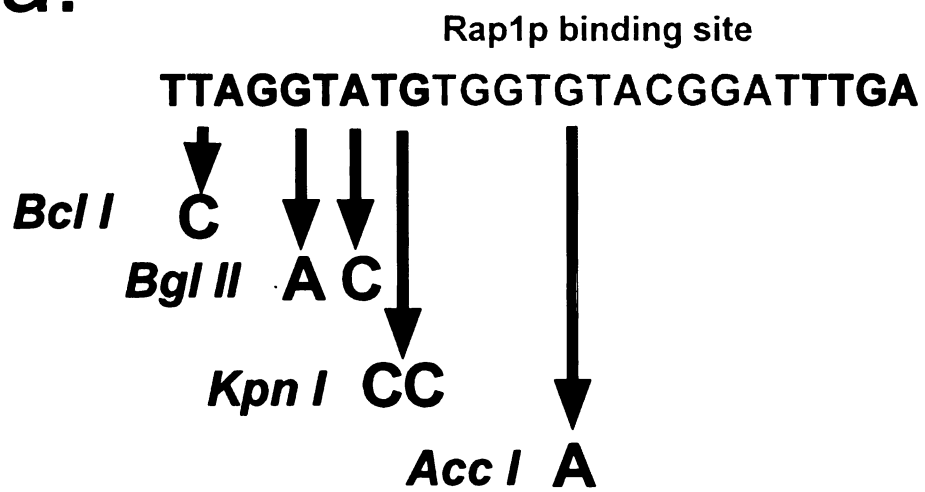
40. Walmsley, R. W., C. S. Chan, B. K. Tye, and T. D. Petes. 1984. Unusual DNA sequences associated with the ends of yeast chromosomes. *Nature.* 310:157-60.

41. Watson, J. D. 1972. Origin of concatomeric T7 DNA. *Nature of New Biology.* 239:197-201.

42. Wotton, D., and D. Shore. 1997. A novel Rap1p-interacting factor, Rif2p, cooperates with Rif1p to regulate telomere length in Saccharomyces cerevisiae. *Genes Dev.* 11:748-60.

43. Yu, G. L., J. D. Bradley, L. D. Attardi, and E. H. Blackburn. 1990. In vivo alteration of telomere sequences and senescence caused by mutated Tetrahymena telomerase RNAs [see comments]. *Nature.* 344:126-32.

a.



b.

Mutant	*In vitro* Rap1p Binding	Telomere Length	
		~150 gen.	>750 gen.
Acc	~ 1%	Long	Long
Kpn	Normal	**Short**	Long
Bgl	~ 200%	**Short**	Long
Bcl	Normal	WT	WT

Figure 1 - The *TER1* template and mutants. (a) The *K. lactis* telomeric repeat sequence. Grey nucleotides represent the Rap1p binding site. Arrows indicate base mutations for *ter1* mutants, which are named by the unique restriction enzyme site they create in the repeat. (b) Summary of the *in vitro* Rap1p binding affinity as a percentage of wild-type and the associated telomere length phenotypes of *ter1* mutants. **Short** telomeres are any length shorter than WT. Long telomeres are longer than the longest WT telomere (~3.5 kb).

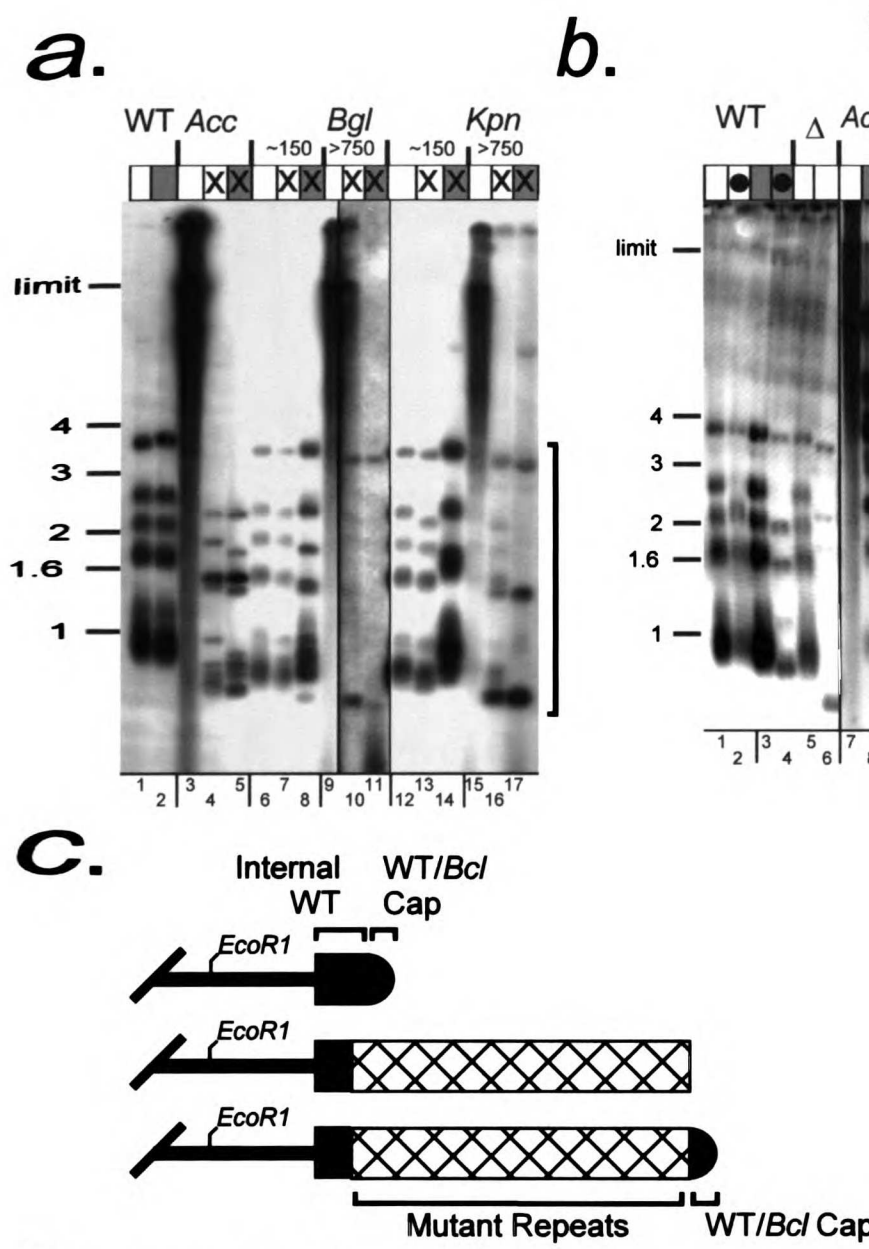


Figure 2 - Southern blot analyses of uncapped and re-capped *ter1* mutants. (a) Analysis of total telomeric tracts and internal wild-type repeats. X's indicate digestion with second enzyme specific to the mutant repeat (i.e. *AccI, BglII, KpnI*). Shaded boxes indicate *ter1-Bcl* re-capped strains. Bracket indicates approximate size range of wild-type telomeres and wild-type repeat tracts remaining in *ter1* mutants. (b) Southern blot analyses of *ter1* strains with and without a *ter1-Bcl* telomere cap. Filled circles indicate digestion with the cap-specific enzyme *BclI*. Shaded boxes indicate re-capped strains. Arrows refer to discrete bands observed in the re-capped strains. (c) Schematic representation of telomeric DNA sequence arrangement. Top to bottom: wild type, uncapped, and re-capped telomeres. Shaded areas: wild-type functioning repeat sequences. Crosshatched boxes: mutant telomeric sequences. Note that the cap can consist of either wild type or functionally wild-type *Bcl* repeats.

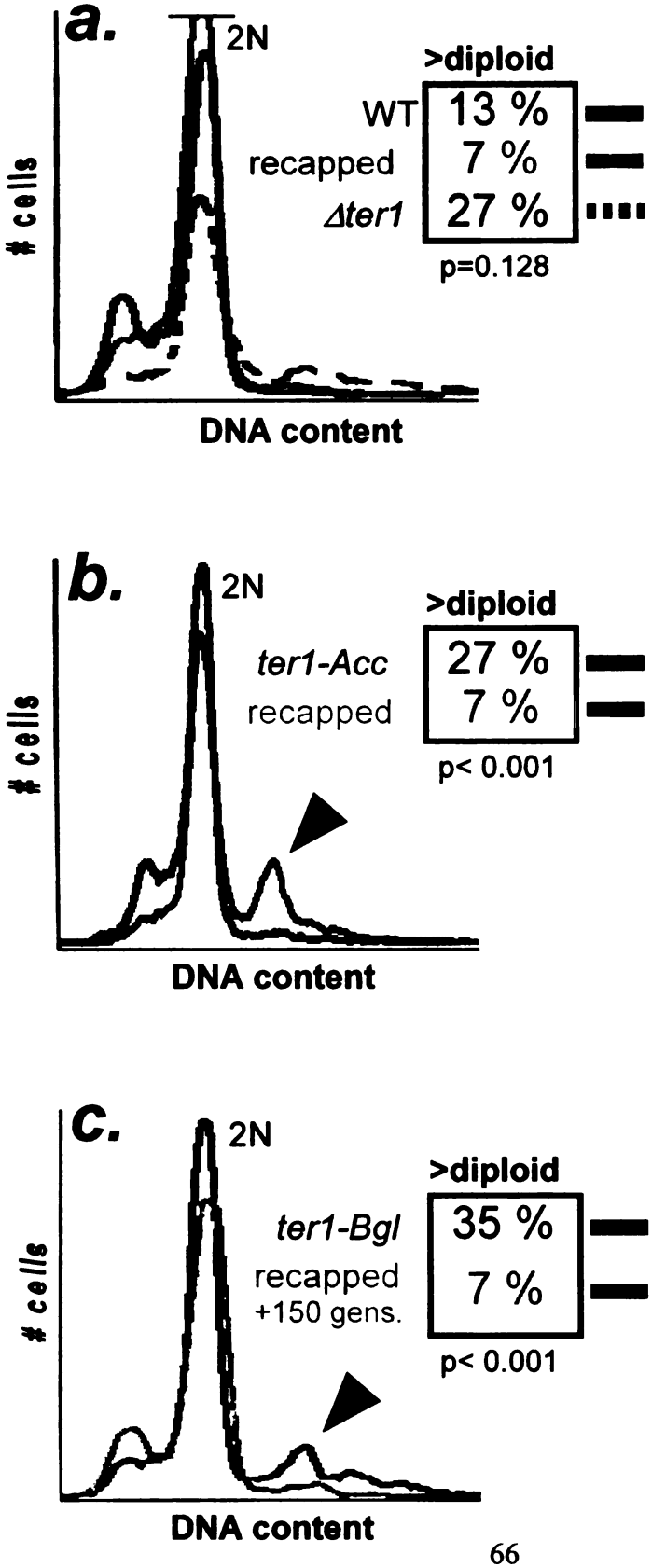


Figure 3 - FACS analysis of uncapped (black histogram) versus re-capped (gray histogram) *TER1* and *Δter1* survivor (dashed histogram) strains (a), *ter1-Acc* (b), *ter1-Bgl* >750 generation (c) strains. Representative plots show number of cells versus propidium iodide signal (>650nm). 2N peak is indicated. Relevant population percentages are shown as rounded averages of at least three replicates. Statistical p-values are from t-tests of data from uncapped versus re-capped populations.

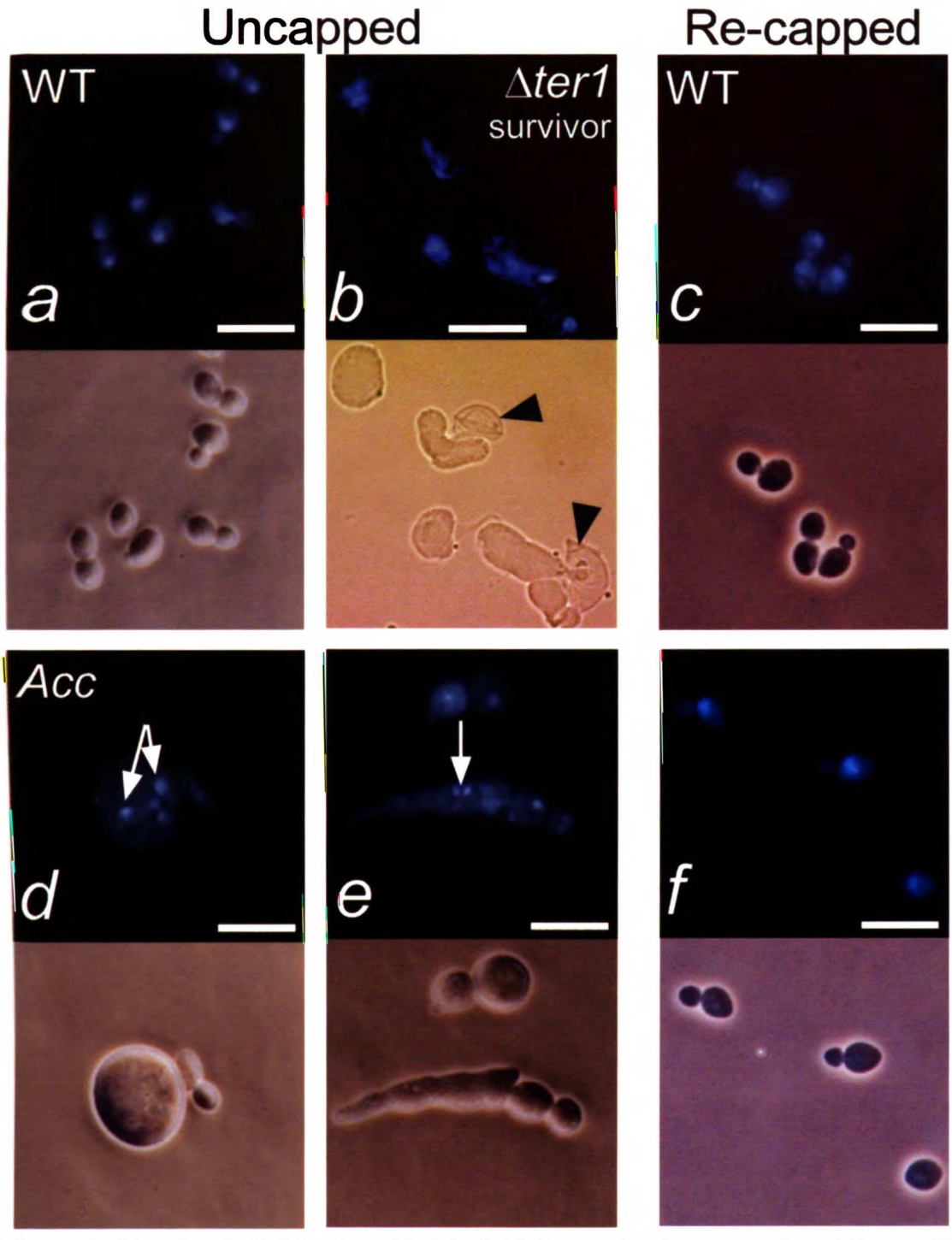


Figure 4 - Panel a, DAPI (top) and brightfield (bottom) microscopy for wild-type *K. lactis* cells. Panel b, post-senescent *Δter1* survivor strain. Panel c, wild type strains re-capped with *ter1-Bcl*. Panels d and e, DAPI and brightfield microscopy showing representative *ter1-Acc* monster cells. Arrows, multiple large DAPI staining structures within a cell body. Panel f, *ter1-Acc* cells re-capped with *ter1-Bcl*. Scale bar, 10 μM.

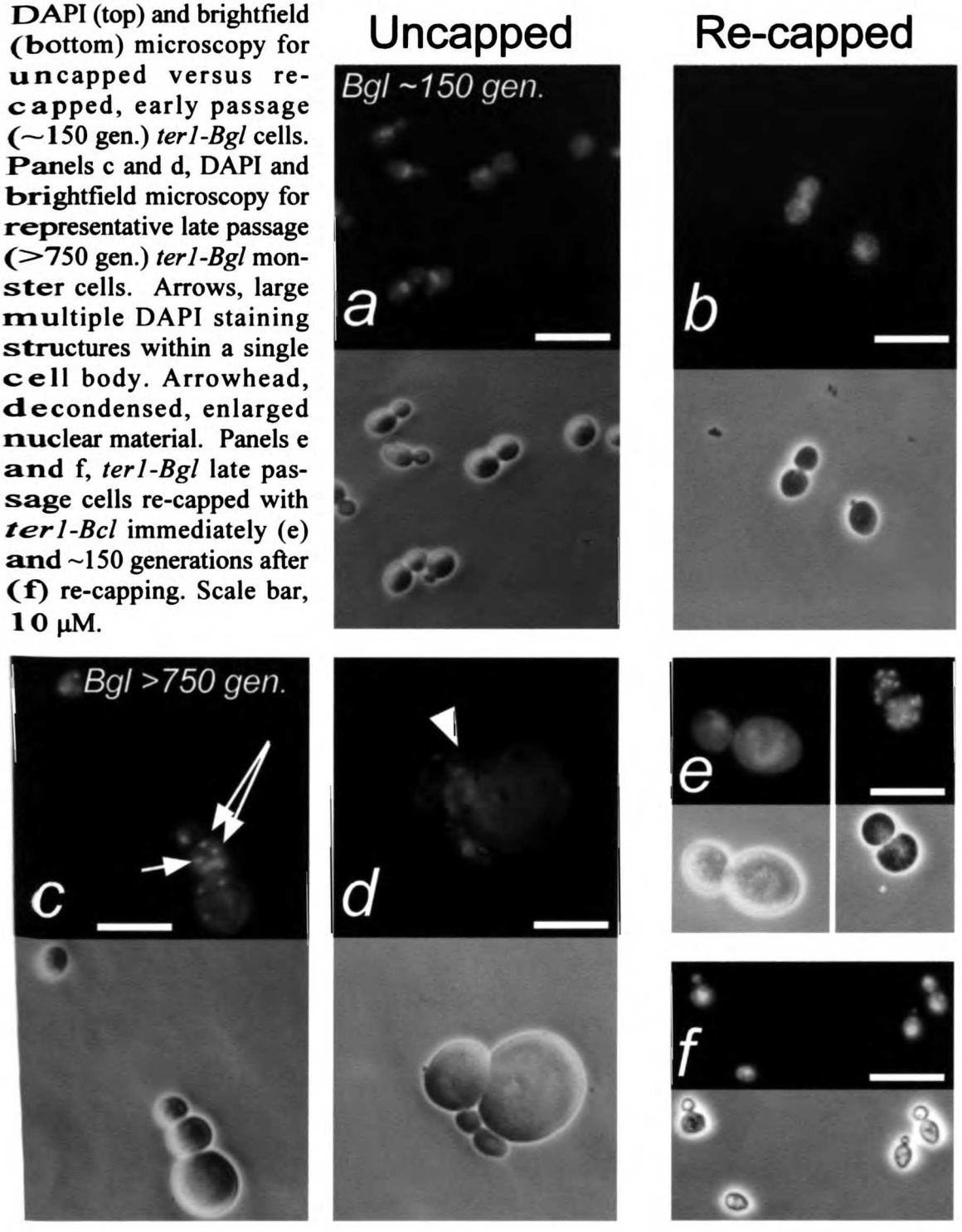


Figure 5- Panels a and b, DAPI (top) and brightfield (bottom) microscopy for uncapped versus re-capped, early passage (~150 gen.) *ter1-Bgl* cells. Panels c and d, DAPI and brightfield microscopy for representative late passage (>750 gen.) *ter1-Bgl* monster cells. Arrows, large multiple DAPI staining structures within a single cell body. Arrowhead, decondensed, enlarged nuclear material. Panels e and f, *ter1-Bgl* late passage cells re-capped with *ter1-Bcl* immediately (e) and ~150 generations after (f) re-capping. Scale bar, 10 μM.

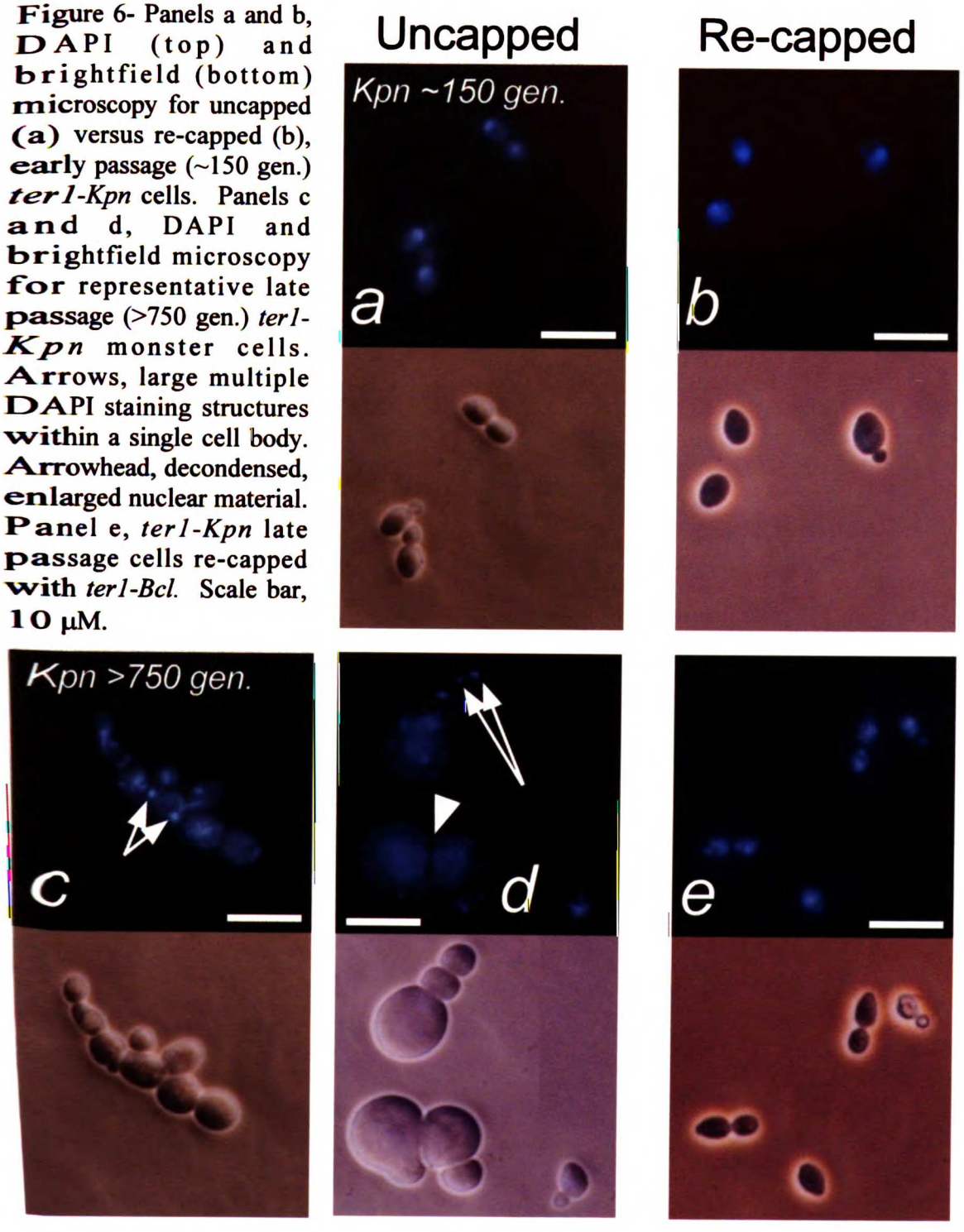


Figure 6- Panels a and b, DAPI (top) and brightfield (bottom) microscopy for uncapped (a) versus re-capped (b), early passage (~150 gen.) *ter1-Kpn* cells. Panels c and d, DAPI and brightfield microscopy for representative late passage (>750 gen.) *ter1-Kpn* monster cells. Arrows, large multiple DAPI staining structures within a single cell body. Arrowhead, decondensed, enlarged nuclear material. Panel e, *ter1-Kpn* late passage cells re-capped with *ter1-Bcl*. Scale bar, 10 μM.

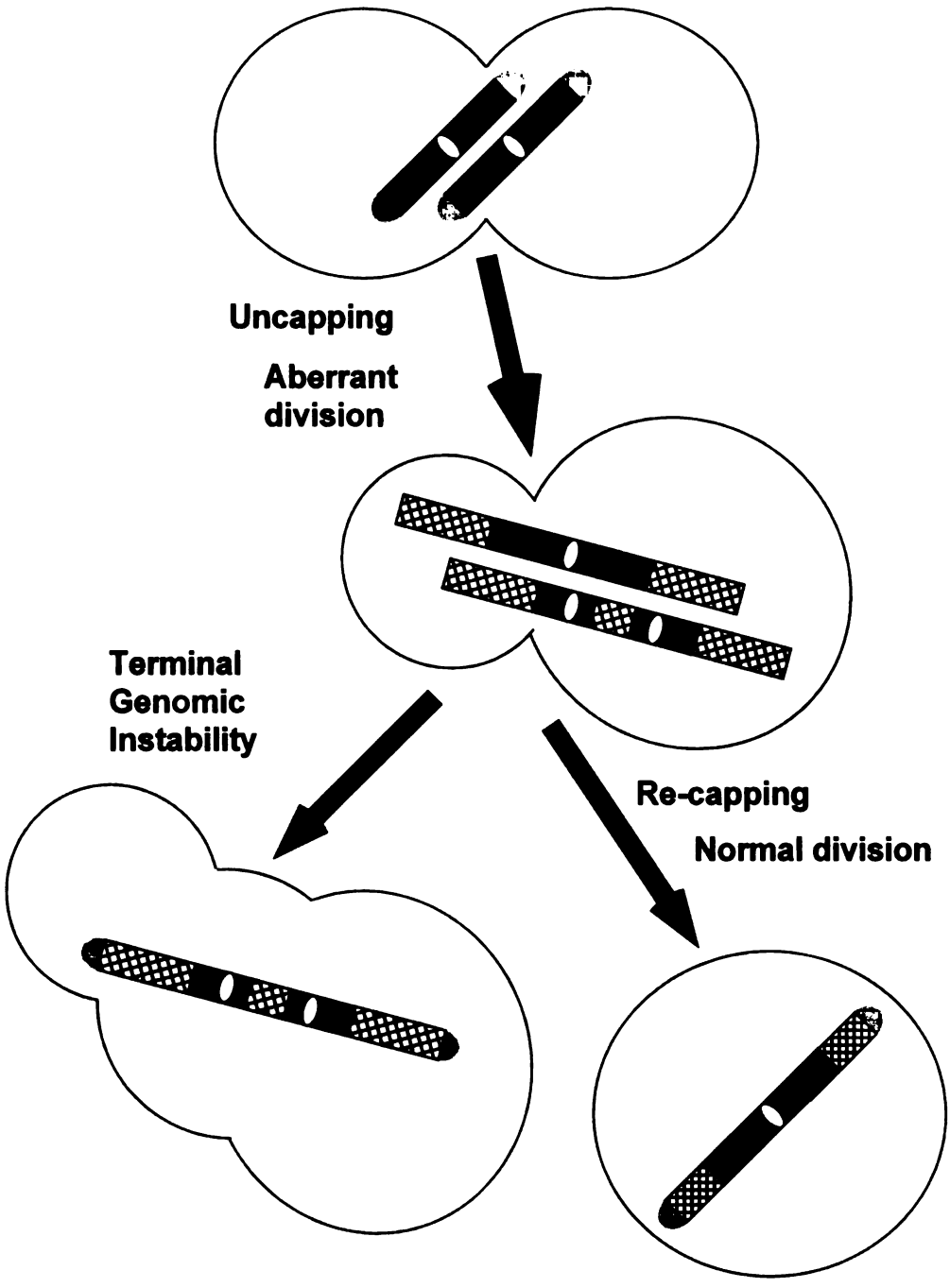


Figure 7- Model for monster cell formation and effects of re-capping. Top, a large budded haploid cell ready for mitosis. One pair of sister chromatids shown. Middle, uncapping leads to chromosome aberrations such as dicentric chromosomes (lower chromosome). Bottom left, segregation of damaged chromosomes leads to terminal monster cells. Bottom right, re-capping of strains with stable chromosomes allows normal division to occur.

I.

I. *TER1* & *Δter1* Budding Indices

	Unbudded	Small Budded	Large Budded	Monster	# cells
WT	22 +/- 7	32 +/- 2	46 +/- 8	**0**	1555
re-capped	19 +/- 1	29 +/- 0.6	52 +/- 0.5	**0.3 +/- 0.6**	299
***Δter1* survivor**	11 +/- 4	28 +/- 3	57 +/- 2	**4 +/- 3**	529
ter1-Acc	15 +/- 0.9	18 +/- 0.7	57 +/- 0.1	**10 +/- 0.1**	701
re-capped	23 +/- 2	29 +/- 5	47 +/- 3	**0**	301

ter1-Bgl	Unbudded	Small Budded	Large Budded	Monster	# cells
~150 gen	18 +/- 3	29 +/- 3	49 +/- 3	**3 +/- 0.7**	709
re-capped	14 +/- 0.7	33 +/- 1	52 +/- 1	**0.3 +/- 0.5**	308
>750 gen.	16 +/- 4	21 +/- 0.4	51 +/- 4	**12 +/- 1**	703
re-capped	8 +/- 2	29 +/- 2	54 +/- 2	**9 +/- 0.5**	302
re-capped +150gen.	27 +/- 3	28 +/- 3	44 +/- 2	**0.9 +/- 0.8**	1153

ter1-Kpn	Unbudded	Small Budded	Large Budded	Monster	# cells
~150 gen.	18 +/- 2	27 +/- 2	53 +/- 2	**3 +/- 0.2**	708
re-capped	16 +/- 3	30 +/- 0.4	53 +/- 2	**0.7 +/- 0.6**	304
>750 gen.	14 +/- 2	20 +/- 0.9	53 +/- 2	**13 +/- 0.9**	943
re-capped	21 +/- 1	28 +/- 2	51 +/- 0.8	**0.7 +/- 0.6**	302

Table 1 - Budding indices of wild-type and *ter1* *K. lactis* strains.
Standard Deviations are shown.
Monster cell populations are in bolded text.
Generations = gens.

Appendix 1

The role of Rap1p-induced DNA bending in *K. lactis*

Introduction

During the course of the experiments performed for Chapter 2, I became interested in the cause for the delayed lengthening phenotype observed for the *ter1-Bgl* and *ter1-Kpn* strains. The *Acc* mutation contains a G to A transition in its core Rap1p binding site, which reduces Rap1p binding affinity 300-fold (Krauskopf and Blackburn 1996) and immediately results in approximately 100-fold increases in telomere length (McEachern and Blackburn 1995). This gross disruption of the telomeric chromatin led to an inability to regulate telomere lengths at discrete sizes and the formation of chromosome fusions (McEachern, Iyer et al. 2000), which lead to polyploidy and genomic instability (McEachern and Blackburn 1995; Smith and Blackburn 1999). These phenotypes were consistent with loss of Rap1p-mediated negative regulation of the chromosome end.

On the other hand, the two other *TER1* template mutations studied in the experiments described in Chapter 2, *ter1-Bgl* and *ter1-Kpn*, were notable because their *in vitro* Rap1p binding was either unchanged or slightly increased (Krauskopf and Blackburn 1996). Yeast strains containing these mutations remained healthy for hundreds of generations, after which they inexplicably and suddenly lost their ability to regulate telomere length or regulation (McEachern and Blackburn 1995; Smith and Blackburn 1999). The mechanism by which these cells lost control remains a mystery since there was no correlation to klRap1p binding. Since factors important for telomere regulation in *S. cerevisiae* had not been cloned in *K. lactis* (except for *klRAP1*) it was difficult to investigate the effects that *Kpn* and *Bgl* repeats had on other factors.

However, the only other factors known to bind directly to *S. cerevisiae* telomeric DNA at the time were Tel2p and the single strand binding protein Cdc13p. Loss of function mutations in *TEL2* resulted in telomeres shortening, not lengthening, and any reduction of Cdc13p binding would also be expected to also result in telomere shortening, since it is required for telomerase recruitment. Also, the considerable delay in telomere lengthening made it difficult to envision how any direct effect on binding could account for the loss of regulation hundred of generations later.

One possibility I investigated was the potential effects of telomeric DNA sequence composition in the ability of klRap1p to bend DNA. It had been well documented in *S. cerevisiae* that Rap1p bends DNA >50 degrees (Muller, Gilson et al. 1994; Konig, Giraldo et al. 1996). I tested the hypothesis that alteration of telomeric DNA sequence composition caused by the *Bgl* and *Kpn* mutations might result in an altered Rap1p DNA bending angle. Other data had shown that a phenotypically silent *TER1* mutant, *ter1-Bcl*, eventually permeated into the internal telomere tracts over many generations (Krauskopf and Blackburn 1998). After 3000 generations *Bcl* repeats could be detected only half way into the WT telomere tract (McEachern, Underwood et al. 2002). If *Kpn* and *Bgl* permeation into the telomere was similar, then gradual encroachment of these repeats could eventually "build-up" enough incorrectly bent DNA to cause the delayed telomere elongation and deregulation observed. To investigate this possibility, I expressed a GST fusion of klRap1p and looked at its ability to bend WT, *Bgl*, and *Kpn* repeats in *in vitro* gel shift assays (Figure 1).

Materials & Methods

Plasmid Construction

Using the pBEND/pGD579X (a pBR322 derivative obtained from Bob Sauer) construct I inserted a single WT, *Bgl*, or *Kpn* telomeric repeat into the *XhoI* site of the multiple cloning site (see Chapter 2, Figure 1 for repeat sequences). Briefly, a single telomere repeat was cloned into a construct with identical restriction sites upstream and downstream of the binding site. Parallel restriction digests are done on the construct using different enzymes. The end result is equal length DNA fragments where the DNA binding site being studied is present at various distances from the end of the restricted DNA. When these "phased" binding sites are used for gel shift analyses with Rap1p, the migration of the protein DNA complex in the gel matrix varies proportionally to the degree of DNA bending induced. By measuring the maximal and minimal migrations of the protein DNA complexes, the angle of DNA bending can be calculated. The relationship is described as: $\cos \alpha/2$ = (distance of slowest migrating band / distance of fastest migrating band).

Protein purification

The klRAP1 gene was cloned in-frame into the pGEX-4T (Pharmacia, NJ) *E. coli* expression construct. An N-terminal GST-fusion protein was induced and purified according to the manufacturer's protocol. Purified protein was either used as GST-RAP1 fusions or was thrombin cleaved to remove the GST according to the manufacturer's specifications before use in gel shift assays. A C-terminally truncated klRAP1 was generated by cutting the pGEX-RAP1 construct with *AflII,* blunting the

ends with Klenow polymerase, and religating them together, creating a premature termination at the 635th codon (Krauskopf and Blackburn 1996). This Rap1ΔC protein was similarly purified for use in gel shift assays.

Gel Shift Assays

Since some of the enzymes used to cut the pBEND fragments produced blunt or 3' overhangs, inefficient end labeling with kinase was a problem in this assay. Therefore, PBEND-WT, *Bgl*, or *Kpn* constructs were amplified by PCR (5'-GGCGTATCACGAGGCCC and 5'-CCAGCAACCGCACCTGT) using standard conditions and were "spiked" with 5 μl of 3000 Ci/mmol [α^{32}-P]dTTP in addition to the standard dNTPs. PCR products were then split equally into 6 tubes, restricted with the appropriate enzyme and Qiagen purified. The enzymes used to make pBEND fragments were *BamHI, EcoRV, BstNI, HindIII, and EcoRI.* This methodology ensured that all DNA fragments were equally labeled. Equal amounts of purified GST-RAP1, GST-RAP1ΔC, or thrombin cleaved RAP1 protein were incubated with pBEND fragments at room temperature for 30 minutes and resolved on non-denaturing polyacrylamide gels. Transferred gel blots were dried and exposed to phosphorimager screens and the distance of RAP1-DNA complex migration from the loading well was determined.

Results & Conclusions

I used pBEND analysis to assess the Rap1p-induced bend angle of WT, *Bgl*, and *Kpn* telomere repeats. Experiments in *S. cerevisiae* suggest that scRap1p bends DNA approximately >50 degrees and that this bend angle is heavily influenced by the N-terminal domain of the protein (Muller, Gilson et al. 1994). I determined that WT, *Bgl*, and *Kpn* are similarly bent by klRap1p and that the angle of DNA bending is between 82 and 92 degrees for all 3 repeat types (Figure 1 A). I also looked at the degree of bending using *RAP1* proteins where the GST moiety was cleaved by thrombin (Figure 1 B) as well as GST-Rap1ΔC (Figure 1 A). Neither cleavage of the GST tag, nor the truncated Rap1p showed significant differences in the degree of DNA bending.

While DNA bending analysis using the pBEND assay suggested that there was no difference between the DNA bend angle of WT, *Bgl* or *Kpn* repeats, these experiments do not rule out that topological differences between these telomeric repeats might contribute to the delayed elongation observed. One limitation of this study was that we only used single telomere repeats in our assays. It is possible that the use of multiple DNA repeats in this assay might result in a different DNA structure. However, pilot experiments using multiple binding sites yielded numerous protein-DNA complexes, which were difficult to clearly resolve and analyze (data not shown). Another possibility is that the binding of another double stranded telomere binding protein may be affected by these mutations or that the single stranded repeats generated by the mutant *ter1-Bgl* or *ter1-Kpn* telomerase have altered binding to other factors that recruit or inhibit telomerase action.

Since the time of these experiments other DNA bending assays have been developed that assess both the 2 dimensional bend angle and the rotation of that bent DNA around a central axis can be measured (Kim, Zwieb et al. 1989). Perhaps measurement by this method might show differences between these DNA repeats. Another possible approach to address DNA topology is to co-crystallize the klRap1p DNA binding domain with the WT, *Bgl*, and *Kpn* repeats. The conditions for the highly homologous scRap1p DNA binding domain have already been determined (Konig and Rhodes 1997), making this experiment quite feasible. Also, the identification homologous proteins in *K. lactis* has been facilitated both by the completion of the *S. cerevisiae* genome, a concerted *K. lactis* genome sequencing effort, and the Genolevures project which used random sequence tagging (RST) to map regions of homology between 13 closely related yeast species (Souciet, Aigle et al. 2000). Perhaps gel shift analyses of *K. lactis* Cdc13p or Tel2p using mutant template repeats might shed more light on why *Bgl* and *Kpn* telomeric repeats have delayed lengthening phenotypes *in vivo*.

References

Kim, J., C. Zwieb, et al. (1989). "Bending of DNA by gene-regulatory proteins: construction and use of a DNA bending vector." Gene **85**(1): 15-23.

Konig, P., R. Giraldo, et al. (1996). "The crystal structure of the DNA-binding domain of yeast RAP1 in complex with telomeric DNA." Cell **85**(1): 125-36.

Konig, P. and D. Rhodes (1997). "Recognition of telomeric DNA." Trends Biochem Sci **22**(2): 43-7.

Krauskopf, A. and E. H. Blackburn (1996). "Control of telomere growth by interactions of RAP1 with the most distal telomeric repeats." Nature **383**(6598): 354-7.

Krauskopf, A. and E. H. Blackburn (1998). "Rap1 protein regulates telomere turnover in yeast." Proc Natl Acad Sci U S A **95**(21): 12486-91.

McEachern, M. J. and E. H. Blackburn (1995). "Runaway telomere elongation caused by telomerase RNA gene mutations." Nature **376**(6539): 403-9.

McEachern, M. J., S. Iyer, et al. (2000). "Telomere fusions caused by mutating the terminal region of telomeric DNA." Proc Natl Acad Sci U S A **97**(21): 11409-14.

McEachern, M. J., D. H. Underwood, et al. (2002). "Dynamics of telomeric DNA turnover in yeast." Genetics, **in press**.

Muller, T., E. Gilson, et al. (1994). "Imaging the asymmetrical DNA bend induced by repressor activator protein 1 with scanning tunneling microscopy." J Struct Biol **113**(1): 1-12.

Smith, C. D. and E. H. Blackburn (1999). "Uncapping and deregulation of telomeres lead to detrimental cellular consequences in yeast." J Cell Biol **145**(2): 203-14.

Souciet, J., M. Aigle, et al. (2000). "Genomic exploration of the hemiascomycetous yeasts: 1. A set of yeast species for molecular evolution studies." FEBS Lett **487**(1): 3-12.

A

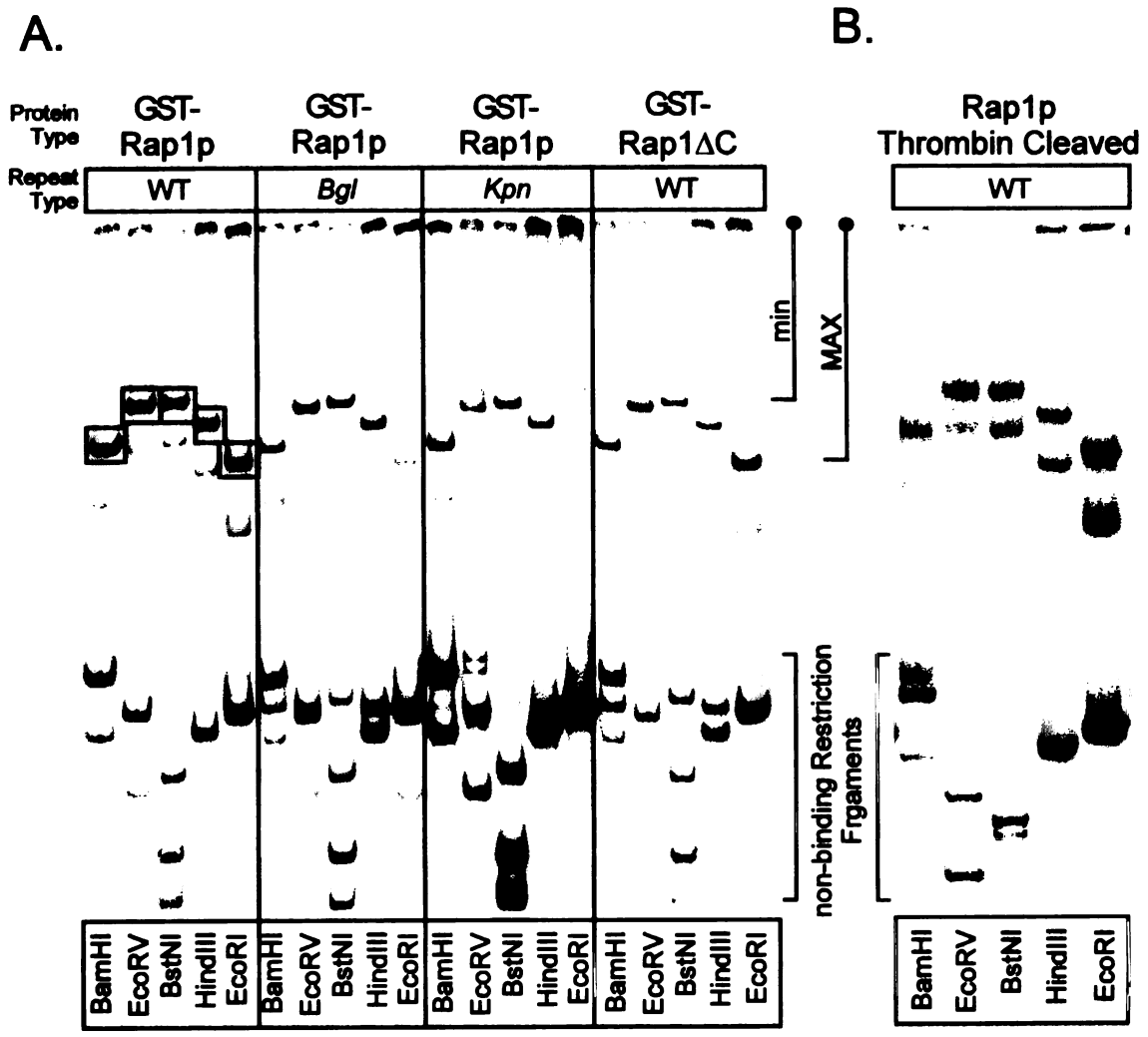
Prote
Ty

Rece
Ty

C.

Figu
tion
locat
GST
(B) w
ed do
bands
botto
brack
indica

shown



C. Bend Angle $\alpha = 2 * \cos^{-1} \frac{\text{(distance of minimal migrating band)}}{\text{(distance of MAXIMAL migrating band)}}$

Figure 1 - *K. lactis* GST-Rap1p was incubated with equally-sized, radio-labeled restriction fragments of the pBEND construct, where distance of the Rap1p binding site was located at varying distances from the end of the fragment. As indicated, either whole GST fusion protein (A), C-terminally truncated GST-Rap1p, or thrombin-cleaved Rap1p (B) was used. The telomeric repeat used in each assay is indicated in boxes at top. Shifted doublet complexes were observed due to degredation of the fusion protein. Boxed bands used for analysis. The restriction fragment used for each lane is shown at the bottom. Fragments from the restriction digest that do not bind Rap1p are indicated by bracket at the bottom. The minimal and maximal migrating bands used for analysis are indicated. The degree of Rap1p-induced DNA bending is determined by the equation shown in C. All repeat and protein types used resulted in an 82^{o}-92^{o} DNA bend.

Appendix 2

The Kinetics of Senescence in

K. lactis

Introduction

In cells that undergo continual division, the maintenance of telomeric DNA is essential to maintain genomic integrity. While mammalian cells can enter quiescent or fully differentiated states where telomeres no longer need active maintenance, constantly dividing cells such as epithelial cells, stem cells, and germ line cells require telomerase activity. Yeast are unicellular organisms that also require constant telomere maintenance. In *S. cerevisiae* and *K. lactis,* telomeres are maintained at a constant length of ~350bp and ~250-500 bp, respectively. When the ability to maintain telomeric DNA is compromised, yeast cells undergo a phenomenon termed senescence. When cells senesce, their telomeres reach a critically short length and the cells go through a period of "crisis". Senescence kills approximately 99% of cells in a population. The small remainder of cells that pass through senescence are termed "survivors" (Lundblad and Blackburn 1993). Survivors arise through a *RAD52*-dependent recombination process that essentially recombines homologous subtelomeric DNA between chromosome ends to maintain a buffer of end sequences that are still competent to protect the chromosome ends. Ablation of *RAD52* in addition to genes that are essential for telomere maintenance generally results in total death of the population. In *S. cerevisiae*, subtelomeric Y' elements are used as the homologous template for recombination-driven telomere maintenance. In *K. lactis*, survivors are generated by a similar *RAD52*-dependent process (McEachern and Blackburn 1996). *K. lactis* does not possess subtelomeric Y' elements, but does possess highly homologous regions in 11 of

12 of their telomeres. The telomeric tracts themselves become elongated in *Δter1* survivors. In addition, subtelomeric recombination events are frequent.

During my investigation of the phenotypic effects of template mutants that resulted in extreme telomere elongation and deregulation (see Chapter 2), I became interested in whether senescent cells might utilize similar cellular pathways. Despite the obvious differences in telomere length between these two experimental treatments, there was considerable similarity between the cellular phenotypes that these two populations exhibited. In this study, I deleted the telomerase RNA gene, *TER1*, in *K. lactis* strains and passaged them regularly until the populations senesced and recovered. I then assessed the colony phenotype, population budding indices, telomere length and degree of regulation, cellular DNA content, and cellular morphology throughout this timecourse. I found striking correlations between colony size, cell cycle arrest time, cellular morphology, and cellular DNA content defects in cells passing through senescence.

Materials & Methods

K. lactis strains were transformed with a truncated *ter1* gene that replaced the WT *TER1* gene and resulted in deletion of the majority of its coding sequence. Transformants were selected for 5-FOA resistance and *ter1* deletion was confirmed by PCR. Positive *Δter1* transformants were passaged on YPD plates every 3 days (each passage equals roughly 30 generations). I passaged multiple transformants (termed *Δter1*#1 and *Δter1*#2), based on the apparent health of the colony at each restreak. The *Δter1* #1 isolate represents the healthiest cells from each restreak, while the *Δter1* #2 isolate represent the sickest looking colony. This was intended to control for the variability of colony sizes in plated populations. Colony morphologies were photographed for each passage. A 20 ml YPD culture was grown for each isolate and split in half. One half was used to prepare genomic DNAs for Southern Blot analyses, and the other half was fixed in 70% ethanol + PBS for later use in microscopic observations and FACS analyses. Southern blots, microscopy, and FACS analyses were performed as described in the Methods section for Chapter 2 (Smith and Blackburn 1999).

Results

A number of interesting changes took place in *Δter1* cultures undergoing senescence. The first observation that I made was that survivors could arise at varying times after the deletion of *TER1*. Indeed, by most of the criteria measured in this study, the *Δter1* #1 isolate seemed to emerge from transformation already in survivor mode. By Southern blot analysis, recombination was activated by the 2nd restreak (Figure 1 C). These cells rarely exhibited the small colony phenotypes (Figure 1 A) that are typical of cells undergoing senescence. The budding profiles of this isolate did not change dramatically over the period of this timecourse either. The one variation that was distinct was a decrease in small budded cells relative to WT through the 200th generation (Figure 1 B). Phenotypically, there was an increase in large or multibudded "monster" cells at the 225th generation (Figure 1 B). About 4% of the *Δter1* #1 population had this phenotype (Figure 2 A). The appearance of these cells was correlated with a slight peak in the greater than 2N DNA content relative to WT (Figure 2 B and 2 C). However, none of the changes in the budding profile or increased DNA content was strongly correlated with the telomere length phenotype or colony morphology. Indeed, the *Δter1* #1 isolate did not seem to exhibit any severe growth defects over the length of the timecourse, other than the emergence of a minor subpopulation of cells with aberrant morphologies.

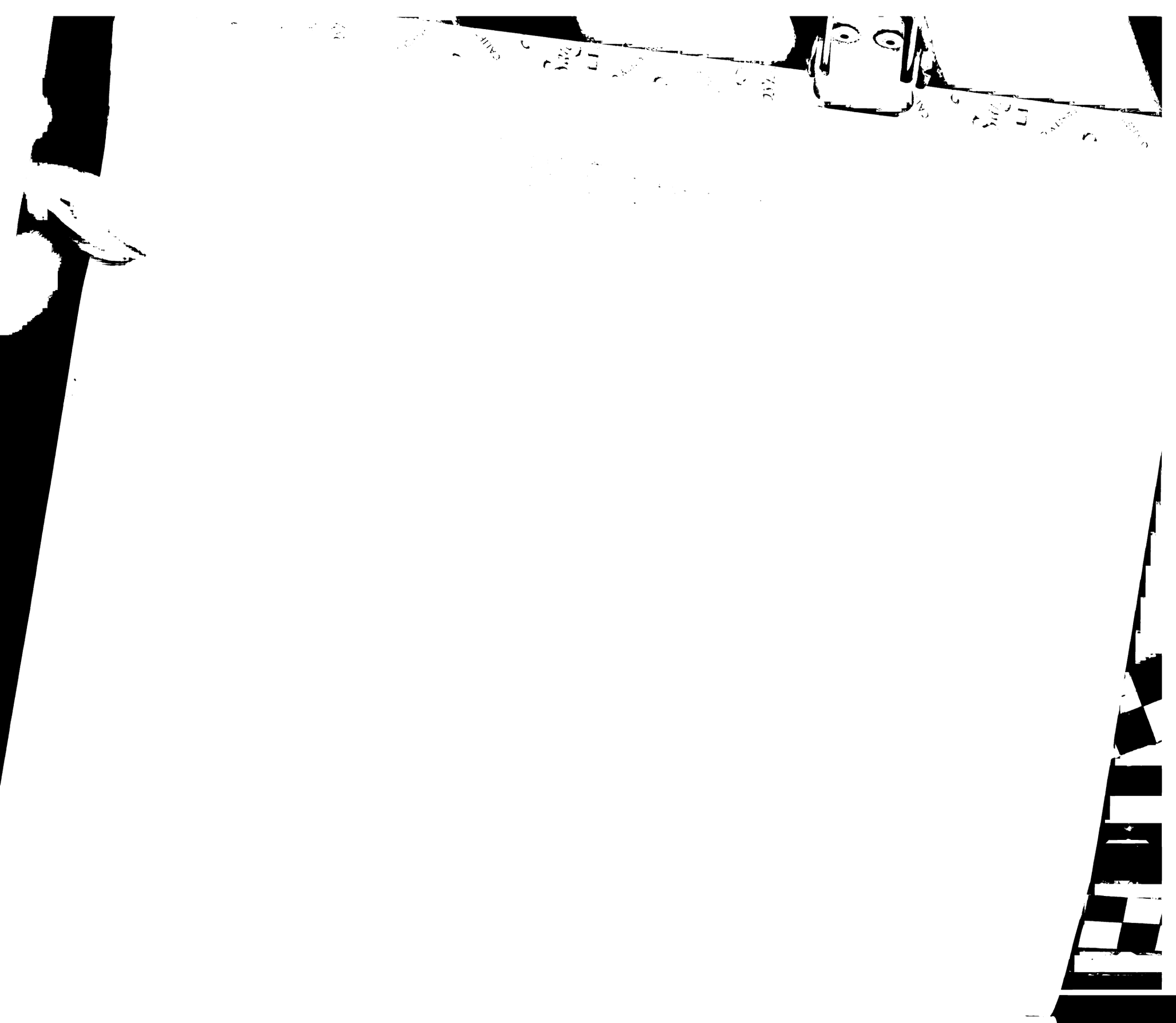
The *Δter1* #2 isolate exhibited markedly different behavior over the timecourse. This isolate showed more a more typical pattern of telomere shortening, senescence and recovery. Colonies from the *Δter1* #2 isolates appeared healthy until the cells

underwent senescence after the 210th generation (Figure 1 A). While the telomeres in this strains were clearly more recombinogenic before senescence (Figure 1 C), during the period of senescence the telomere length was quite short and overall telomeric signal was low on Southern blots. There was a dramatic change in the budding index profile of this isolate as the telomeres reached maximal shortness. As cells underwent senescence there was a significant decrease in unbudded and small budded cells (Figure 1 B). These changes are indicative of a cell cycle arrest in very late S-phase or G2. Once survivors arose from senescence and recombinational telomere maintenance was in effect, the colony morphology and budding index phenotypes disappeared (Figure 1 A and 1 B). Coincident with the period of senescence and minimal telomere length, was a correlated with an increased subpopulation of morphologically aberrant cells (Figure 1 B, 2 A). While these cells only represented 5% of the population, as judged by budding indices, populations of senescent *Δter1* #2 cells showed a dramatic increase in cellular DNA content (Figure 2 B). At approximately the 245th generation (the peak of senescence), 29% of the *Δter1* #2 population had DNA content that was greater than the diploid amount (Figure 2 C). There was a simultaneous decrease in cells with 1N and 2N DNA content at this time. These results support budding index data that suggested a G2 arrest.

As the timecourse progressed I noticed that the cell walls of the extensively passaged *Δter1* survivors looked degraded and irregular (Figure 2 A). While there was no change in the DNA content, viability, colony morphology, or telomere pattern in *Δter1* survivors that had been passaged extensively, the apparent progressive degradation of the cell was notable. The appearance of the degraded cell wall

phenotype also occurred in earlier isolates, but the frequency of this phenotype seemed to increase with time.

As a final experimental manipulation of *Δter1* survivors, I performed the "re-capping" experiment (Figure 3). This procedure involved retransforming a functionally WT telomerase RNA into the *Δter1* strains. As had been observed on strains with other length regulation defects, the reintroduction of *TER1* resulted in a complete reversal of all of the detrimental phenotypes associated with deletion of the *TER1* gene. Other than changes in the sizes of their telomeric tracts and changes that resulted from recombination in subtelomeric regions, re-capped *Δter1* survivor populations had WT viability and morphology (Figure 3). Interestingly, one feature by which WT and re-capped *Δter1* strains differed was the higher fraction of cells with 2N DNA content compared to the WT control, which had a relatively larger 1N subpopulation of cells (Figure 2 C and 3 B).



Conclusions

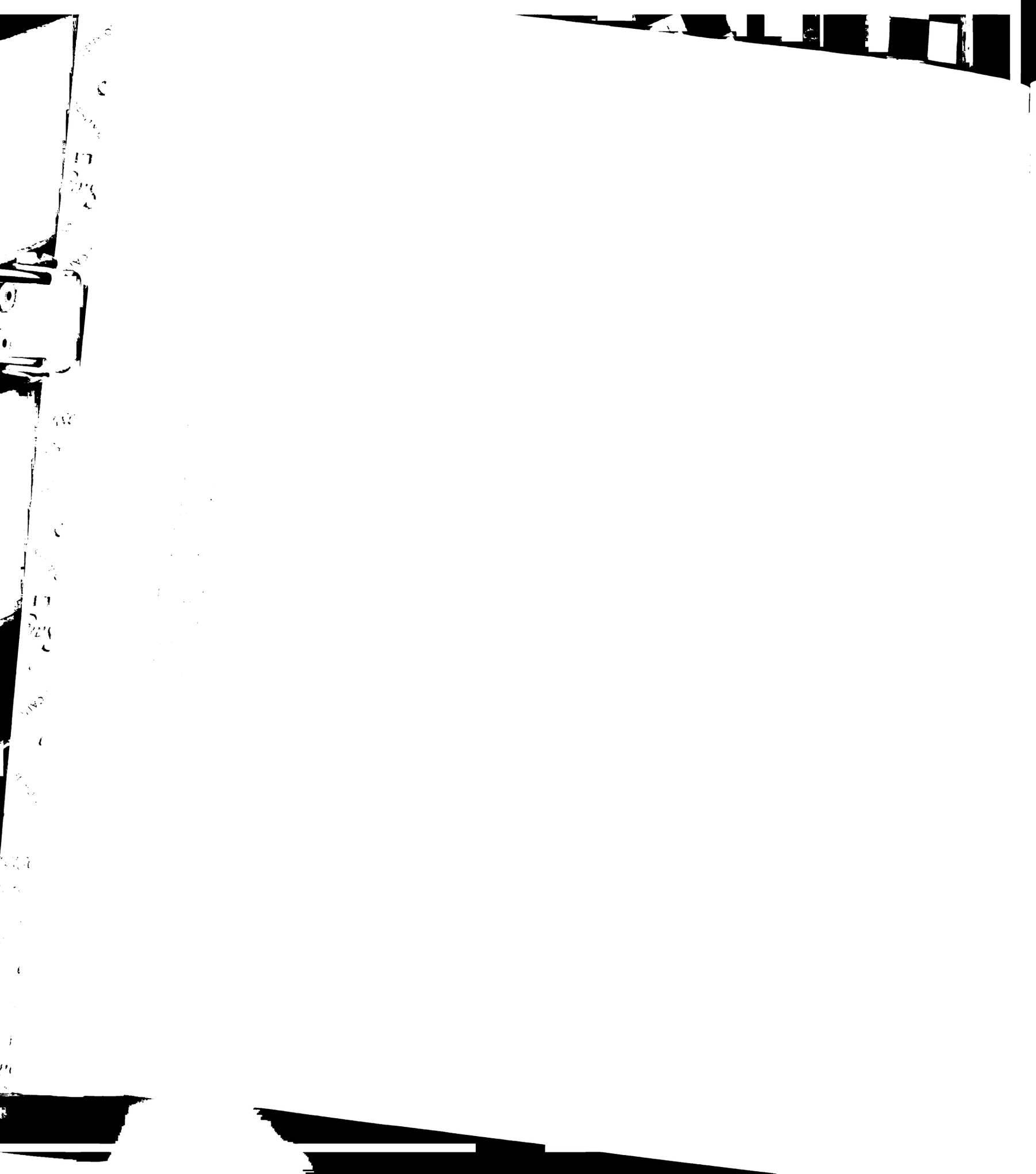
These studies were undertaken to explore the similarities and differences between the "sickness" resulting from loss of telomerase function and the "sickness" resulting from the loss of telomere length regulation described in Chapter 2. While it was well known that loss of telomerase function in yeast resulted in a "crisis" event once telomeres became critically short and that "survivor" populations arose in *RAD52*-dependent process, the actual phenotypes and cell cycle defect of cells with shortening telomeres had not been reported.

While most *Δter1* transformants underwent the typical telomere shortening and crisis events that are well known to be associated with senescence, the *Δter1*#1 isolates in this study appeared to have entered survivor mode almost immediately. Other than minor increases in multibudded "monster" cells and a slight increase in the greater than diploid DNA content, this isolate appeared essentially wild type. In short, recombinational telomere maintenance was sufficient to allow the *Δter1*#1 cell population to propagate. Since these cells never experienced critically shortened telomere tracts, they did not experience any other detrimental aspects associated with senescence.

In contrast, the *Δter1*#2 isolate experienced severe population crises over the period of this timecourse. When telomere tracts in this strain became short there was a highly correlated cell cycle arrest at G2/M. At this stage there was a significant decrease in small budded and unbudded cells, and a significant increase in large budded cells. Also, approximately one third of the population exhibited an increase in DNA

content that was in excess of the diploid genome. These results suggest that the crisis associated with senescence is typified by a G2/M arrest in the cell cycle. Perhaps, since short telomeres lack sufficient binding sites for Rap1p, they are incapable of recruiting a repressive chromatin complex that prevents recognition of the chromosome end as double-strand break and then results in a DNA damage arrest. Once gene conversion or recombination is able to restore telomere tracts, the cells are capable of proceeding through the cell cycle once again.

Recent results in *S. cerevisiae* have shed some light onto the phenotypes I observed in senescing and survivor *K. lactis* strains. Genetic dissection of survivor formation has shown that the *RAD50* and *RAD51* genes are involved in 2 classes of survivor formation. Type I survivor are typified by increased Y' recombination and is dependent upon the *RAD51* gene while Type II survivor formation is dependent upon the *RAD50* gene (Teng, Chang et al. 2000). Type II survivors do not show increased Y' recombination, but do show dramatic increases in telomere tract length that become progressively shorter over time. Once a short length is reached, the cells reenter the type II survivor mode to replenish telomere tracts. It has been suggested that when faced with a critically short telomere, cells undergo Type I recombination until they amplify the telomere tracts associate with Y' elements. Once there is enough of a "template" of telomeric DNA to support Type II recombination, this becomes the preferred mode of telomere maintenance. It is possible, however, for cells to enter immediately into Type II recombination. Perhaps the *Δter1*#1 isolate in this study entered directly into a Type-II-like mode of survival and this avoided the cellular phenotypes and checkpoint associated with short telomeres.



References

Lundblad, V. and E. H. Blackburn (1993). "An alternative pathway for yeast telomere maintenance rescues est1- senescence." Cell **73**(2): 347-60.

McEachern, M. J. and E. H. Blackburn (1996). "Cap-prevented recombination between terminal telomeric repeat arrays (telomere CPR) maintains telomeres in Kluyveromyces lactis lacking telomerase." Genes Dev **10**(14): 1822-34.

Smith, C. D. and E. H. Blackburn (1999). "Uncapping and deregulation of telomeres lead to detrimental cellular consequences in yeast." J Cell Biol **145**(2): 203-14.

Teng, S. C., J. Chang, et al. (2000). "Telomerase-independent lengthening of yeast telomeres occurs by an abrupt Rad50p-dependent, Rif-inhibited recombinational process." Mol Cell **6**(4): 947-52.

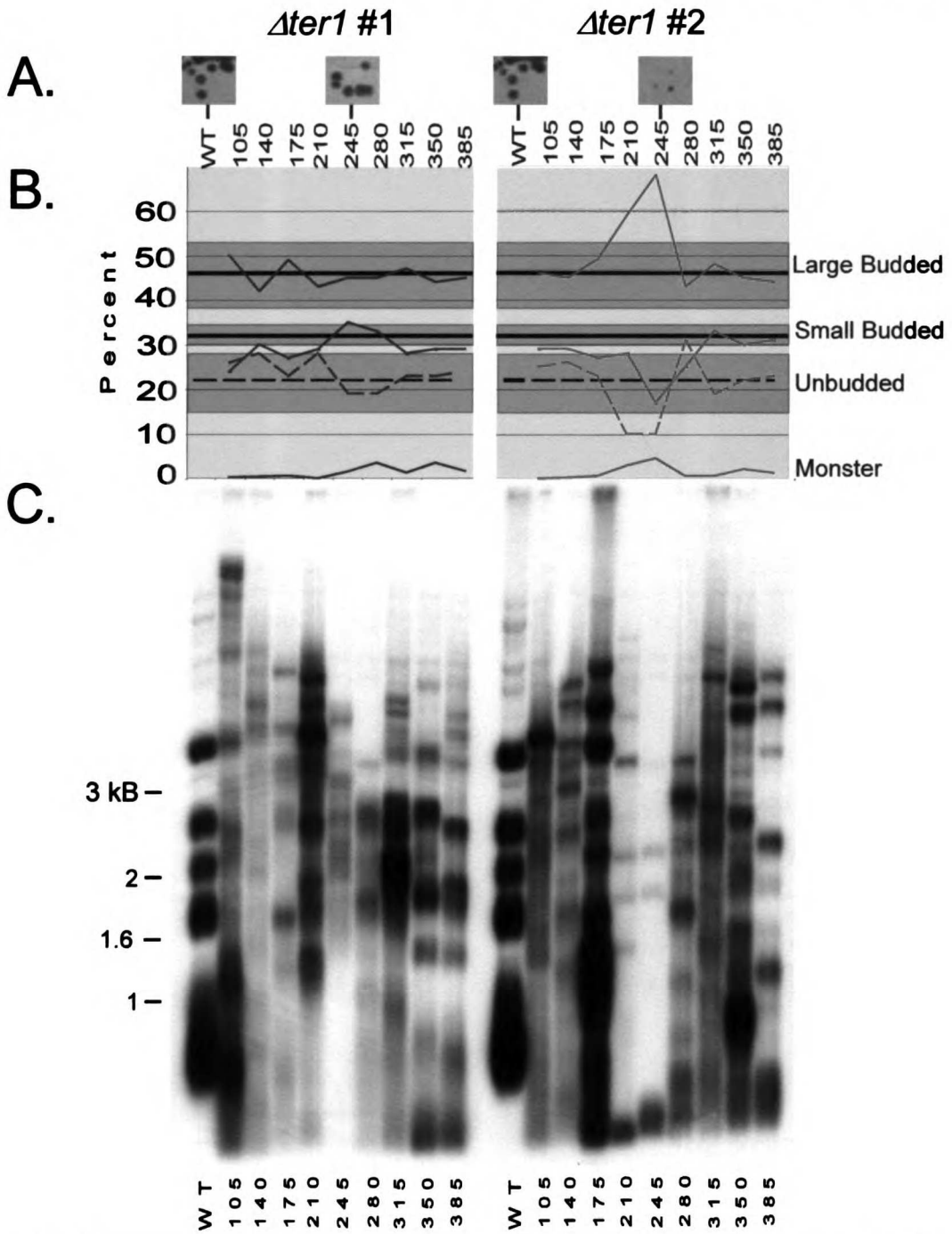


Figure 1 - A timecourse of senescence for 2 *Δter1 K. lactis* isolates. Colony morphologies are shown for the healthiest and sickest timepoint of the timecourse (A). Budding indices are shown for the timecourses in B. The average value for WT is shown by dark black lines and the range for WT is shown by dark grey areas. WT cultures typically do not have any monster cells. The telomere profile for both isolates are shown in C. The number of generations after *TER1* deletion are shown at the bottom and top. The approximate size of markers for the telomere bands is shown at the left.

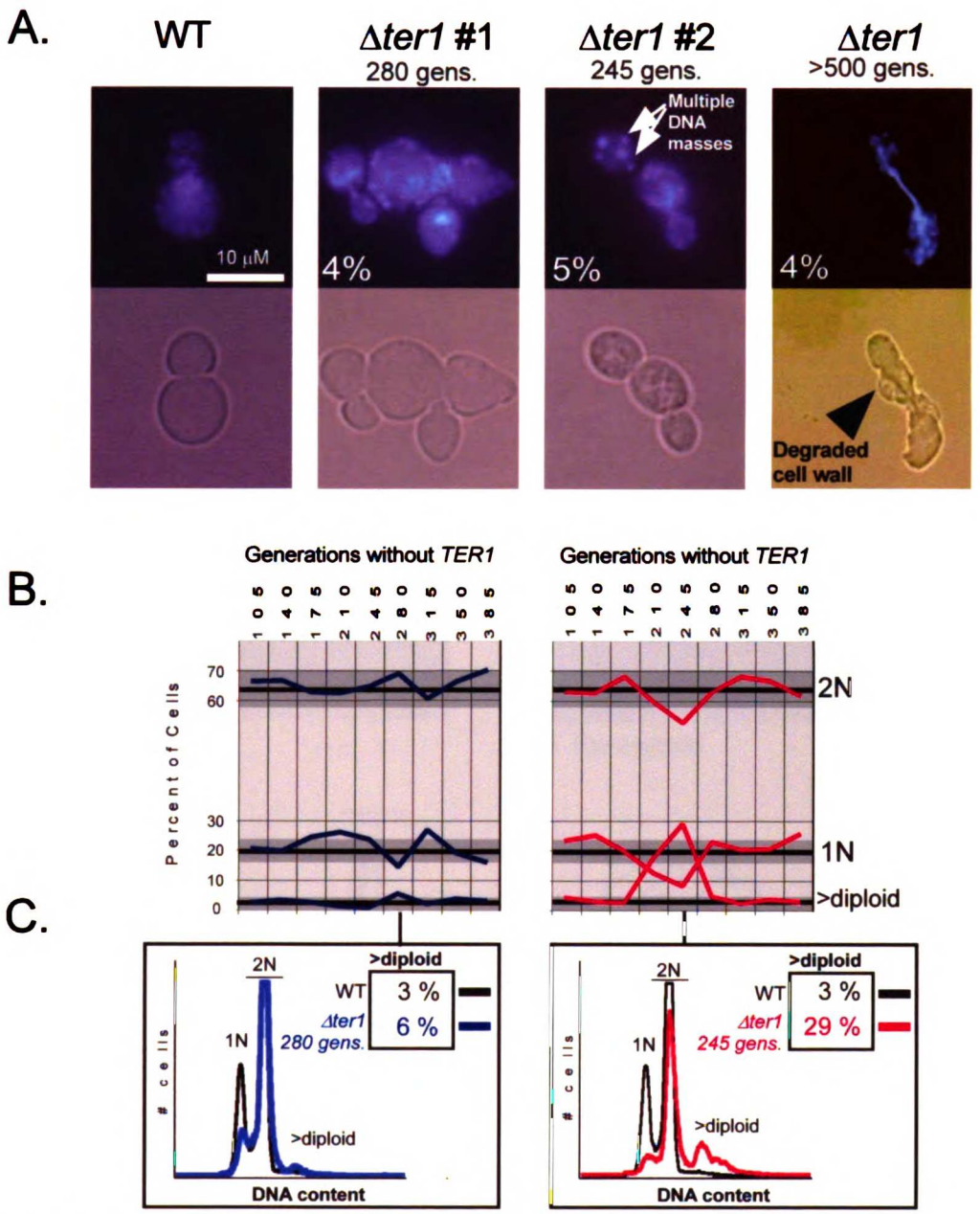


Figure 2 - Aberrant cellular morphologies and DNA content analysis for 2 *Δter1* isolates. Cellular morphologies are shown for *Δter1* islolates #1 and #2 at their sickest timepoints as well as a late-passage post-senescent survivor (A). DNA content profiles are shown for the timecourses in B. The average value for WT is shown by dark black lines and the range for WT is show by dark grey areas. Representaive FACS profiles for isolates #1 (blue) and #2 (red) at their sickest timepoint shown in C. 1N, 2N, and greater than diploid DNA content are indicated. The percentage of greater than diploid DNA content is shown in boxes. The number of generations after deletion of *TER1* are shown at the top of B.

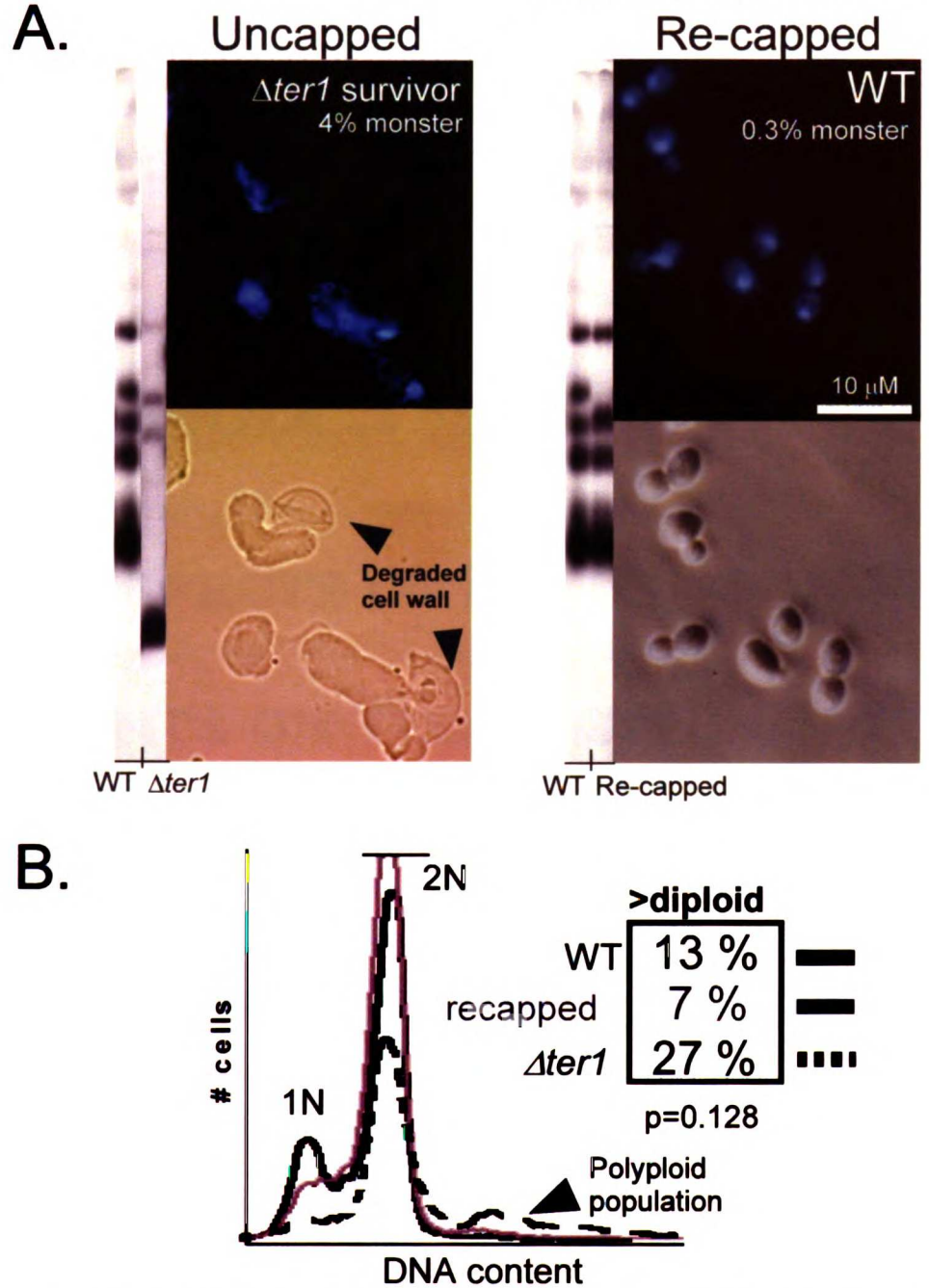


Figure 3 - Recapping of *Δter1* isolates results in phenotypic revesion to WT. Cellular morphologies are shown for late passage *Δter1* islolates before and after recapping with a functionally WT *TER1* (A). The telomere length phenotypes of WT and *Δter1* are shown in the lefty panel, while the telomere length phenotypes of re-capped strains are shown in the right panel. DNA content FACS profiles are shown for the WT, *Δter1*, and re-capped *Δter1* strains in B. The 1N and 2N DNA peaks are indicated. The greater than diploid, polyploid population is indicated by arrowhead. The percentage of greater than diploid DNA content before and after re-capping is shown in boxes.

Appendix 3

The Effects of Putative CDC Mutants on Telomere Length in *K. lactis*

Introduction

Telomere replication and maintenance is intrinsically linked to the cell cycle. For example, telomere replication occurs specifically in late S-phase-G2 in the budding yeast S cerevisiae (Diede and Gottschling 1999; Marcand, Brevet et al. 2000). The *RAP1* and *SIR* gene products, which comprise a major portion of telomeric chromatin, also have cell cycle specific duties. Rap1 is required for carbohydrate metabolism and protein translation functions that occur in the early stages of the cell cycle (Lieb, Liu et al. 2001) and delocalized from telomeres during mitosis (Laroche, Martin et al. 2000). SIR2 is required for rDNA silencing which is correlated with rDNA chromatin condensation late in the cell cycle (Guacci, Hogan et al. 1994). The SIR proteins also relocalize to DNA damage during replication and prior to chromosome segregation (Martin, Laroche et al. 1999; Mills, Sinclair et al. 1999; Laroche, Martin et al. 2000). Furthermore, telomeres acquire an overhang specifically in S phase (Dionne and Wellinger 1996). This overhang is bound by Cdc13p, which plays a major role in telomerase recruitment and telomere maintenance (Evans and Lundblad 1999; Pennock, Buckley et al. 2001).

A number of genes involved in telomere maintenance have alleles that result in arrests at various cell cycle stages (Carson and Hartwell 1985). For example, the *cdc13-1* allele results in a G2/M arrest and the generation of long single strand telomeric DNA tracts (Garvik, Carson et al. 1995). Similarly, the *TEL1* and *TEL2* genes were isolated by screening the existing Hartwell cell-cycle-division (CDC) mutant library (Hartwell, Culotti et al. 1974) for genes that arrested at the non-permissive temperature

and also resulted in telomere length phenotypes. Both *TEL1* and *TEL2* have shorter than WT telomere lengths (Lustig and Petes 1986).

Results & Conclusions

A screen similar to the Hartwell screen was undertaken by Linda Silveira in *K. lactis*. She generated a bank of temperature sensitive (ts) mutants that had normal cell cycle kinetics at the permissive temperature (25°C), but halted in the cell cycle at the non-permissive temperature (36°C). This collection was generated by EMS mutagenesis and was in the process of being backcrossed when I undertook this project. Essentially, I performed the same assay that Thomas Petes did and decided to screen Linda's ts-library for genes that might lengthen or shorten telomeres at the non-permissive temperature. Although the strains were not fully backcrossed, which makes it difficult to attribute phenotypes to a single locus, I decided to screen them anyway, hoping that any interesting results might help prioritize which genes merited further study. The assay that I used was to simply look at telomere length at both the permissive and non-permissive temperatures by Southern blot analysis. I screened 63 putative-ts mutants by Southern blots analysis, but did not find any that looked like they had a reproducibly significant effect on telomere lengths. A representative sample of mutants is shown in Figure 1.

Since the time that I undertook this experiments, significant work has been done on this library. The library now has 400 mutants, 9 of which have strong *CDC* phenotypes. All of these mutants are blocked at the large budded stage. Some of them are multibudded or have elongated buds that are reminiscent of the *K. lactis* monster

cells we observed in *ter1* template mutant strains. Although complementation groups have not been determined, all of these mutants have now been backcrossed extensively (Linda Silveira, personal communication). It will be of great interest to see if these more characterize *CDC* mutants have telomere phenotypes and if so, if they correspond to known genes or novel ones.

References

Carson, M. J. and L. Hartwell (1985). "CDC17: an essential gene that prevents telomere elongation in yeast." Cell **42**(1): 249-57.

Diede, S. J. and D. E. Gottschling (1999). "Telomerase-mediated telomere addition in vivo requires DNA primase and DNA polymerases alpha and delta." Cell **99**(7): 723-33.

Dionne, I. and R. J. Wellinger (1996). "Cell cycle-regulated generation of single-stranded G-rich DNA in the absence of telomerase." Proc Natl Acad Sci U S A **93**(24): 13902-7.

Evans, S. K. and V. Lundblad (1999). "Est1 and Cdc13 as comediators of telomerase access." Science **286**(5437): 117-20.

Garvik, B., M. Carson, et al. (1995). "Single-stranded DNA arising at telomeres in cdc13 mutants may constitute a specific signal for the RAD9 checkpoint." Mol Cell Biol **15**(11): 6128-38.

Guacci, V., E. Hogan, et al. (1994). "Chromosome condensation and sister chromatid pairing in budding yeast." J Cell Biol **125**(3): 517-30.

Hartwell, L. H., J. Culotti, et al. (1974). "Genetic control of the cell division cycle in yeast." Science **183**(120): 46-51.

Laroche, T., S. G. Martin, et al. (2000). "The dynamics of yeast telomeres and silencing proteins through the cell cycle." J Struct Biol **129**(2-3): 159-74.

Lieb, J. D., X. Liu, et al. (2001). "Promoter-specific binding of Rap1 revealed by genome-wide maps of protein-DNA association." Nat Genet **28**(4): 327-34.

Lustig, A. J. and T. D. Petes (1986). "Identification of yeast mutants with altered telomere structure." Proc Natl Acad Sci U S A **83**(5): 1398-402.

Marcand, S., V. Brevet, et al. (2000). "Cell cycle restriction of telomere elongation." Curr Biol **10**(8): 487-90.

Martin, S. G., T. Laroche, et al. (1999). "Relocalization of telomeric Ku and SIR proteins in response to DNA strand breaks in yeast." Cell **97**(5): 621-33.

Mills, K. D., D. A. Sinclair, et al. (1999). "MEC1-dependent redistribution of the Sir3 silencing protein from telomeres to DNA double-strand breaks." Cell **97**(5): 609-20.

Pennock, E., K. Buckley, et al. (2001). "Cdc13 delivers separate complexes to the telomere for end protection and replication." Cell **104**(3): 387-96.

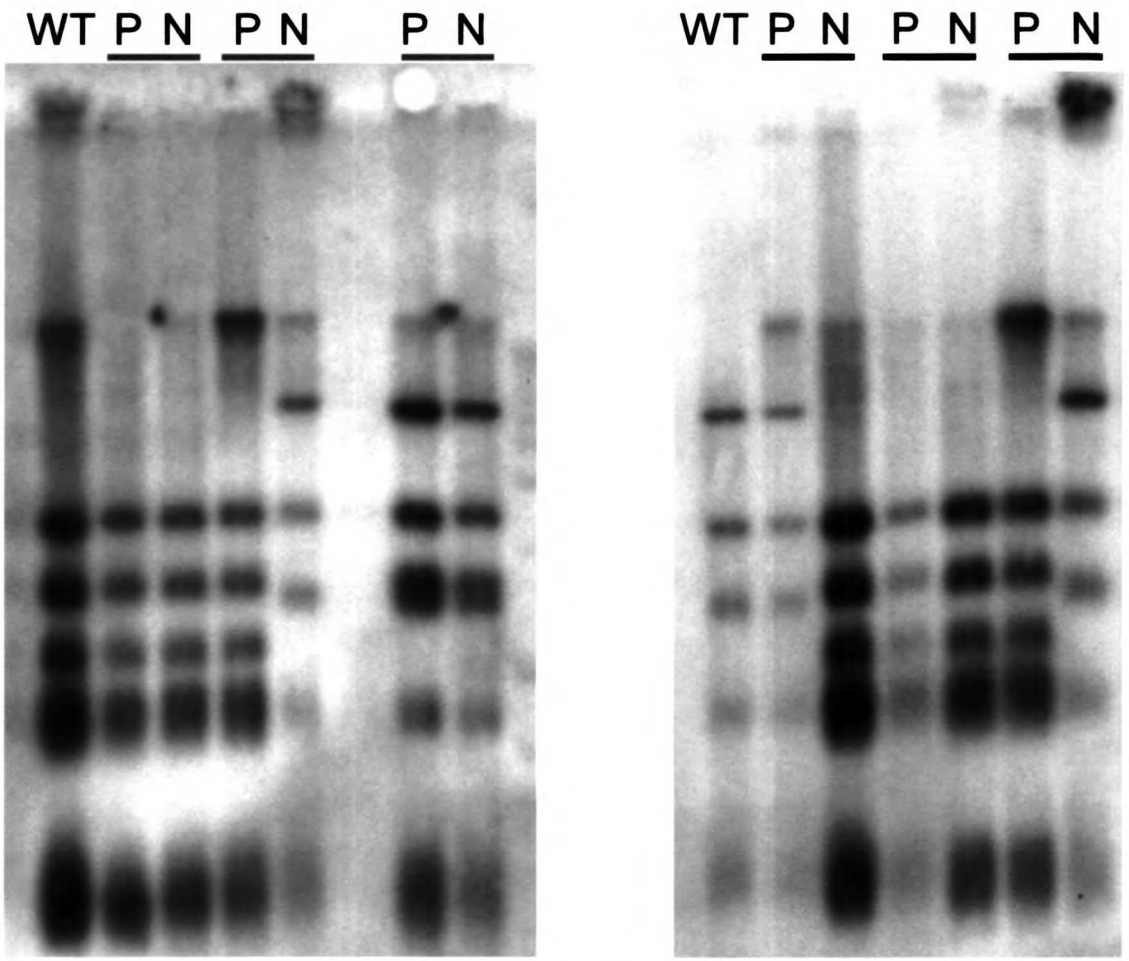


Figure 1 - A sample of 63 mutagenized *K.lactis* strains that show growth at the permissive (P) temperature (25°C), but no growth at the non-permissive (N) temperature (36°C). None of the 63 putative CDC mutants that were screened exhibited increased telomere deregulation, elongation, or telomere shortening by Southern blot analysis with probes to the *K.lactis* telomeric repeat sequence.

Chapter 3

Telomeric Chromatin Remodeling Through the Cell Cycle in *S. cerevisiae*

When you get to the end of your rope, tie a knot and hang on.
Franklin D. Roosevelt, quoted Kansas City Star, June 5, 1977

Abstract

In *S. cerevisiae*, telomeric DNA is protected by non-nucleosomal chromatin, tethered by the Rap1 protein. Factors interacting with Rap1p, such as Rif and Sir proteins, are thought to have further interactions with conventional nucleosomic chromatin to create a repressive structure that protects the chromosome end. While the end-protection function of telomeres is essential for genomic stability, telomeric DNA must also be copied by the conventional DNA replication machinery and replenished by telomerase, suggesting that transient remodeling of the telomeric chromatin might result in distinct protein complexes at different stages of the cell cycle. Using chromatin immunoprecipitation, we monitored the association of Rap1p, Rif1p, Rif2p, and the protein component of telomerase, Est2p, with telomeric DNA through the cell cycle. We provide evidence for dynamic remodeling of these telomeric components, which is correlated with an unknown modification of the Rif2 protein. Microarray analysis also shows that Rif1p association with the chromosome ends extends to subtelomeric regions far internal to the terminal telomeric repeats and correlates strongly with the previously determined genomic footprints of Rap1p and the Sir2-4 proteins in these regions.

Introduction

Telomeres are non-nucleosomal protein-DNA complexes that prevent uncontrolled fusion, degradation, recombination, and elongation of chromosome ends (Muller 1938; McClintock 1941; Wright, Gottschling et al. 1992; Sandell and Zakian 1993; Hande, Samper et al. 1999; Smith and Blackburn 1999). In *S. cerevisiae*, terminal telomeric DNA is composed of ~350 base pairs (bp) of short, degenerate TG_{1-3} repeats. One telomeric strand is polymerized by telomerase in late S-phase through G2 of the cell cycle (Cohn and Blackburn 1995; Diede and Gottschling 1999; Marcand, Brevet et al. 2000), and forms an S-phase specific TG_{1-3} overhang (Wellinger, Wolf et al. 1993; Wellinger, Ethier et al. 1996). The conventional DNA polymerase machinery is thought to synthesize the complementary $C_{1-3}A$ strand. Duplex telomeric DNA repeats are bound by the sequence-specific binding protein Rap1p, which recruits the Rif1 and Rif2 proteins as well as the Sir3 and Sir4 proteins via its C-terminal domain (Moretti, Freeman et al. 1994; Moazed and Johnson 1996; Moretti and Shore 2001).

Telomeric regions in yeast also contain subtelomeric DNA sequences called X- and Y' elements (Chan and Tye 1983). X-elements are not highly homologous to each other and exist at all chromosome ends. Y' elements fall into 2 size classes, 5.2 and 6.7 kilobases (kB), and are present at about half of the chromosome ends next to the terminal TG_{1-3} tracts (Louis and Haber 1990; Louis and Haber 1992). All Y' elements contain an open reading frame (ORF) which encodes a protein with *in vitro* helicase activity (Yamada, Hayatsu et al. 1998). The Y' elements are also bounded by short (~150 bp) tracts of telomeric DNA. Since Y' elements often occur in tandem arrays of 2-4 repeats, these short stretches of telomeric DNA are found internal to the

chromosome ends at distances depending upon the size class and number of Y' elements present at a given end.

Prior studies have shown that Rap1p, Ku, and the Sir2-4 proteins are crosslinkable to DNA as far in as 3-15 kB from the chromosome end, consistent with simultaneous binding to both telomeric DNA at chromosome ends and internal Y' repeats (Hecht, Strahl-Bolsinger et al. 1996; Strahl-Bolsinger, Hecht et al. 1997; Martin, Laroche et al. 1999; Lieb, Liu et al. 2001). Recent evidence that Sir3p may simultaneously associate via different sub-domains with Rap1p, Sir4p, and histones H3 and H4 suggests that Rap1p may spread over this large region both through direct sequence specific binding to telomeric DNA repeats and through protein-protein interactions with Sir3p or Sir4p which are spread into the Y' elements (Moretti and Shore 2001)(Moretti, Freeman et al. 1994; Cockell, Palladino et al. 1995). One model of telomeric chromatin posits that the Rap1p and Sir proteins bound to the terminal telomeric tracts "fold back" to interact with internal histones, creating a higher order protective complex at the chromosome end (Grunstein 1997).

The Rif1 and Rif2 proteins, which negatively regulate telomere length (Hardy, Sussel et al. 1992; Wotton and Shore 1997), are also tethered to telomeric DNA through interactions with one another and the Rap1p C-terminus. Since deletion of the Rif proteins has a more pronounced effect on telomere length than SIR3 or SIR4 deletion, it has been suggested that the Rif proteins interact with the most distal Rap1p molecules on the terminal telomeric $TG_{1\text{-}3}$ tracts, while the Sir2-4 proteins interact with more internal Rap1p molecules (Wotton and Shore 1997). However, it remains unknown what combinations of Rif and Sir proteins bind to the Rap1p C-terminus *in vivo* or when

in the cell cycle these associations may occur. While Rif1- and Rif2-transactivator fusion proteins are capable of associating with internal tracts of telomeric DNA linked to a HIS3 reporter gene (Bourns, Alexander et al. 1998), it has not been determined whether Rif proteins are present on the internal Y' telomeric tracts of native telomeres. If so, these internal telomeric tracts could effectively extend telomeric chromatin significantly into the chromosome end region beyond the terminal telomeric tracts.

While protection of chromosome ends through repressive chromatin is an important function of telomeres, it is also essential for long-term cell division that telomeric DNA be replenished by telomerase. Polymerization by telomerase has been observed in the late S-phase and G2/M phases of the cell cycle (Diede and Gottschling 1999; Marcand, Brevet et al. 2000), while chromosome end replication from late-activating origins is thought to occur starting from mid-late S-phase in the cell cycle (Raghuraman, Winzeler et al. 2001). A number of factors are required for telomere maintenance *in vivo*. Telomerase action minimally requires the core telomerase components Est2p and the TLC1 RNA, Cdc13p (Nugent, Bosco et al. 1998), Stn1p (Grandin, Damon et al. 2001; Pennock, Buckley et al. 2001), Est1p (Evans and Lundblad 1999; Pennock, Buckley et al. 2001), Est3p (Hughes, Evans et al. 2000), Polα, Polδ (Diede and Gottschling 1999), and is promoted by the MRE11, RAD50, XRS2 (MRX) complex (Lendvay, Morris et al. 1996; Nugent, Bosco et al. 1998, Ritchie, 2000 #40; Diede and Gottschling 1999; Ritchie and Petes 2000; Diede and Gottschling 2001; Tsukamoto, Taggart et al. 2001) and the Ku proteins (Peterson, Stellwagen et al. 2001).

It is probable that repressive telomeric chromatin, regardless of whether it includes interactions with internal Y' telomeric repeats or nucleosomal regions, would

need to be transiently displaced to allow telomere replication to occur. In the simplest model, the negative regulators of telomere length (i.e. Rap1p, Rif1p, and Rif2p) would be present at chromosome ends at times when the positive regulators, such as telomerase, were not. Accordingly, we investigated the ability of Rap1p, Rif1p, Rif2p,and Est2p to immunoprecipitate telomeric DNA through the cell cycle. We found that all four of these telomere components studied were crosslinkable to telomeric DNA, and that this association changed significantly through the cell cycle. We also investigated the distance to which Rif1p binding extends in from chromosome ends, and determined that it extends many kilobases (kB) in from the terminal telomere TG_{1-3} tracts. These data suggest that telomeric chromatin is actively remodeled through the cell cycle and may involve long-range interactions over many kB of the chromosomal end region.

Materials & Methods

Strain Construction

Strain genotypes are listed in the Supplementary Data. The S288C yeast strain BY4736 was obtained from ATCC (Manassas, VA)(Brachmann, Davies et al. 1998). Isogenic derivatives of BY4736 were used for all chromatin immunoprecipitation and microarray experiments. Epitope-tagged and deletion strains were generated using homologous PCR recombination (Longtine, McKenzie et al. 1998). All epitope-tagged genes were constructed to retain their endogenous promoters. EST2 was 13xMYC epitope tagged using existing constructs (Longtine, McKenzie et al. 1998), while RIF1 and RIF2 were "-proA" epitope tagged with two Z-domains (Nilsson, Moks et al. 1987) from protein A (gift of Dennis Wykoff). Briefly, the F_c-binding Z-domain of protein A was duplicated in tandem and cloned into pFA6a in frame to yield a C-terminal tagging vector. Transformants were screened by PCR and Western blot analyses.

Plasmids containing *tlc1-476A* mutation (Chan, Chang et al. 2001) were transformed into epitope-tagged BY4736 strains. All strains containing the *tlc1-476A* mutation used in this study were heteroallelic for the *TLC1* locus and also contained a wild-type (WT) copy of the *TLC1* gene. The W303-1a and RIF1-9xMYC strains used in this study were obtained from David Shore (Mishra and Shore 1999). SIR2 deletion plasmids were the generous gift of Danesh Moazed, the *ubc9-1* allele (Seufert, Futcher et al. 1995) was obtained from Kent Duncan, and *TEL1 MEC1 SML1* deletion strains were generated in this lab (Chan, Chang et al. 2001). Mating, sporulation, and tetrad dissection were done according to standard methods (Fink 1991).

Southern Blotting and Hybridization Conditions

Genomic DNAs for Southern blots were prepared as previously described (Chan, Chang et al. 2001). Blots were cross-linked with 1200 μjoules (Stratagene, LaJolla, CA) and hybridized with a γ-^{32}P-labeled telomeric $(TG_3TG)_4$ oligo at 55°C for at least 6 hours. Blots were washed twice and exposed to either phosphoscreens (Molecular Dynamics, Sunnyvale, CA) or Kodak Biomax film (Rochester, NY). Exposures were taken in the linear range and analyzed with Imagequant software (Molecular Dynamics, Sunnyvale, CA).

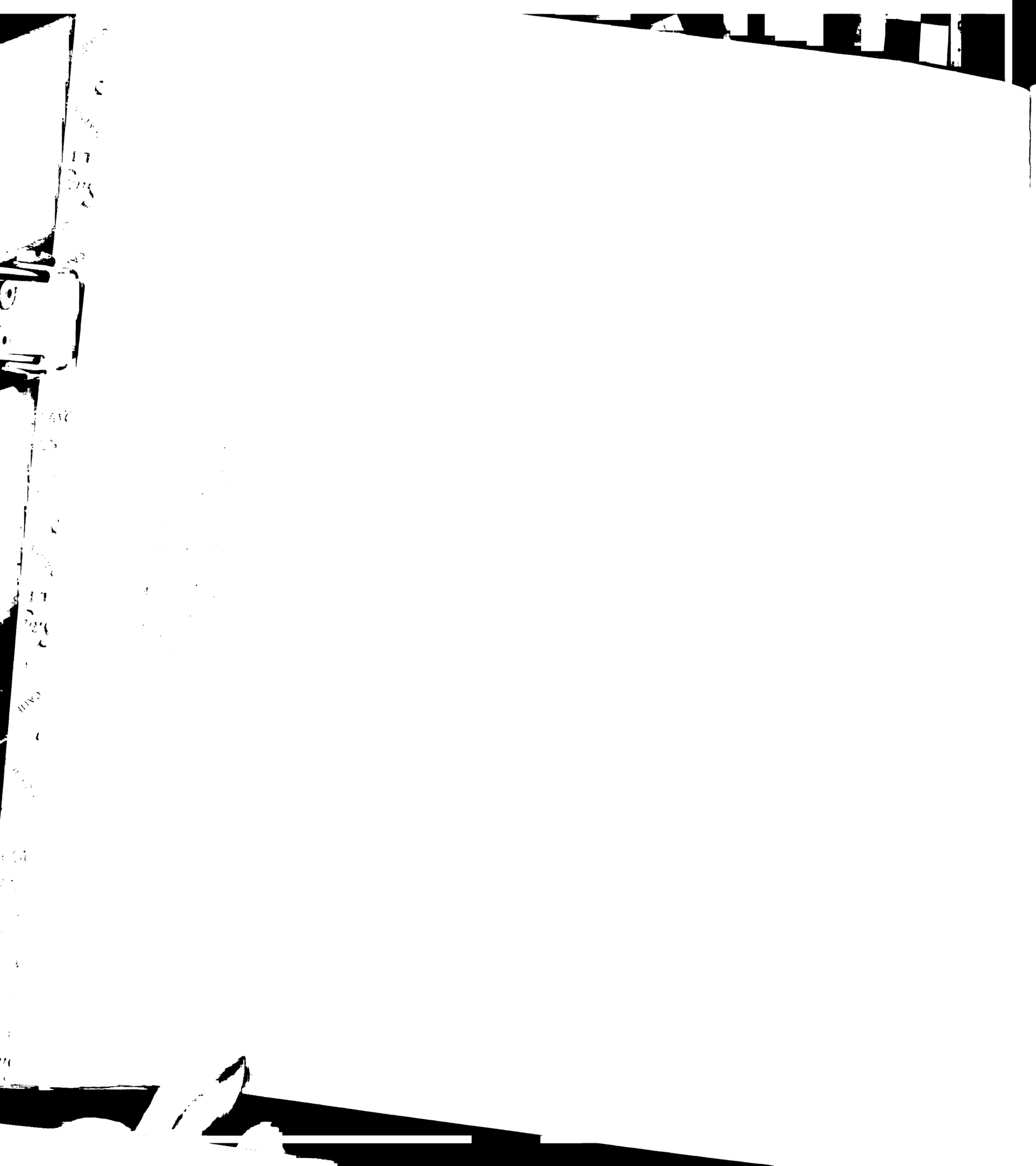
Chromatin Immunoprecipitation & Analysis

Chromatin immunoprecipitations (ChIPs) were performed essentially as described (Hecht, Strahl-Bolsinger et al. 1996; Strahl-Bolsinger, Hecht et al. 1997; Lieb, Liu et al. 2001). For timecourses, 3 or more independent 1.7 liter yeast cultures were grown to 0.3-0.4 A_{600} and arrested for 3.5 hours with 1 μg/ml alpha factor (Biosynthesis Inc, Lewisville, TX). Arrests were confirmed by light microscopy and cultures were then washed twice in an equal culture volume of fresh YPD and released into the cell cycle. Cells were then sampled at 20 minute timepoints for 2 hours and fixed immediately with 1% formaldehyde (Sigma, St. Louis, MO). Cell pellets from 200 ml of culture were resuspended in 1ml of lysis buffer plus protease inhibitors (Roche, Indianapolis, IN) and lysed using a bead beater (Biospec Products Inc, Bartlesville, OK) 3 times for 1 minute at 4°C. Lysates were sonicated (Branson Ultrasonics, Danbury, CT) 3 times for 15 seconds (constant output, 1.5 duty cycle) to a mean DNA length of 300 bp-1kB. DNA lengths were confirmed by agarose electrophoresis, ethidium

staining, and Southern blot analysis. Clarified lysates were immunoprecipitated (IP'd) at 4°C with 50-75 μl of IgG sepharose (Pharmacia, Peapack, NJ) for -proA tagged components, approximately 30 μg of anti-MYC 9E10 (Covance, Princeton, NJ) with 50-75 μl of protein A sepharose (Sigma, St. Louis, MO), or 1:25 dilution of α-RAP1 antibody (Santa Cruz Biotech, Santa Cruz, CA) with 50-75 μl of protein G sepharose (Sigma, St. Louis, MO). Rap1p IP's were also done using a 1:150 dilution of a rabbit polyclonal antibody to Rap1p (Enomoto, McCune-Zierath et al. 1997)(Generous gift of Judith Berman) with 50-75 μl of protein A sepharose. Typically 90% of a given cell lysate was used for ChIPs, while 10% was set aside and designated as "input". IP's were washed as described (Strahl-Bolsinger, Hecht et al. 1997) and de-crosslinked at 65°C for at least 6 hours. De-crosslinked DNA was Qiaquick (Qiagen, Valencia, CA) purified, eluted into 100 μl of buffer.

ChIP samples and their matched input dilutions were denatured in 1.5M NaCl, 0.5N NaOH for 15 minutes at room temperature and applied to a MiniFold-I dot blotter (Schleicher & Schuell, Germany). Typically 75% of ChIP elutions were loaded per timepoint, while 2.5% - 10% of input elutions were loaded. Wells were rinsed alternately with 2 volumes of 3x SSC and denaturing solution. Blots were crosslinked, hybridized, washed, and exposed as described (see above).

For analyses, the "raw" amount of telomeric DNA precipitated for each protein and timepoint was determined by integrating the radioactive hybridization signals from dotblots with Imagequant. The raw ChIP signals determined each protein and timepoint were compared to the raw signal of matched input samples that had been diluted linearly. Raw ChIP and input signals were then divided by the percentage of lysate used



to obtain them (i.e. typically 90% for ChIPs, 10% for inputs) and the percentage of the elution that was applied to the dotblot (i.e. 75% for ChIPs, 2.5%-10% for inputs) so that the "raw percent of input" statistic refers to the percent of telomeric DNA precipitated from the total telomeric DNA in a cell lysate. For example, "Raw Percent of Input" = (Raw Chip Signal / (90% x 75%)) / (Raw Input Signal / (10% x 2.5%)). Over a given timecourse, the "raw percent of input" values for each of the timepoints were normalized to the average value for all of the timepoints in the timecourse to give unbiased "fold change" information over the cell cycle. Est2p and Rif2p timecourse data was also normalized to the raw percent of input values from mock ChIPs (i.e. without antibody) or from untagged control strains for Supplemental Figure B.

Chromatin Spread Immunofluorescence

Duplicate yeast cultures were arrested in α-factor and released as described above. Timepoints were kept on ice for the duration of the time-course and prepared simultaneously. Chromosome spreads were prepared as described (Loidl, Nairz et al. 1991; Michaelis, Ciosk et al. 1997; Biggins, Bhalla et al. 2001). Samples were blocked with PBS + 1% BSA and incubated overnight with pre-cleared primary antibodies overnight at room temperature. The mouse α-MYC 9E10 (Covance, Princeton, NJ) and rabbit α-RAP1 (Enomoto & Berman 97 GD) primary antibodies were used at 1:1000 dilutions. Samples were washed for 5 minutes in PBS twice and covered with 1:1000 goat α-mouse-FITC or mouse α-rabbit-Cy3 conjugated secondary antibodies (Jackson Immunochemicals, W. Grove, PA) for 1 hour at room temperature. Samples were

washed twice in PBS, stained with 1 μg/ml DAPI for 5 minutes and mounted with 90% glycerol, 1 mg/ml phenylenediamine pH 9 (Sigma, St. Louis, MO).

Slides were visualized on a Nikon E600 microscope at 100x magnification and at least 10 fields were captured using a Coolsnap FX CCD (Roper Scientific, Tucson, AZ) for each timepoint. Approximately 75 DAPI-staining, spread nuclei were counted per timepoint. Fields were pseudo-colored blue for DAPI, red for Cy3, and green for FITC in Adobe Photoshop. The total number of discernible Cy3- and FITC-staining foci per spread nucleus were counted for both timecourses. The number of colocalized Cy3- and FITC-staining foci per spread nuclei was also determined. For timecourse analyses, data were processed similar to ChIP data (see above).

Western Blotting Analysis

Lysates were prepared from timecourse cultures similar to ChIPs as described above, except for fixation and sonication. Generally 50-100 ml of mid-log phase yeast cultures were taken to prepare lysates for Western blotting analyses. Immunoprecipitations were performed as described and resolved on SDS-PAGE gels. For detection of the –proA epitope, transferred blots were incubated with either 1:1000 purified rabbit IgG (Jackson Immunochemicals, W. Grove, PA) or 1:1000 polyclonal rabbit α-RIF2 (Generous gift of Jason Lieb) followed by 1:5000 donkey α-rabbit-HRP (Jackson Immunochemicals, W. Grove, PA), and visualized using ECL Plus (Pharmacia, Peapack, NJ). Multiple exposures of the Rif2-proA doublet were taken so that both bands could be analyzed in the linear signal range for Figure 6.

Microarray Production & Analysis

Microarrays were prepared as described (Gerton, DeRisi et al. 2000; Iyer, Horak et al. 2001; Lieb, Liu et al. 2001). Protocols for microarray preparation, hybridizations, ChIP amplification, and fluorescent dye coupling were those described and available from http://www.microarrays.org. Briefly, microarrays containing fragments homologous to all yeast ORFs and intergenic regions were hybridized to ChIP samples from 3 independent, asynchronous Rif1-proA cultures. A common reference sample of amplified BY4736 genomic DNA was used as a control hybridization for all experiments. All genomic features (i.e. ORFs and intergenic fragments) were subjected to median rank analysis (Iyer, Horak et al. 2001; Lieb, Liu et al. 2001). Genomic features whose median percentile rank was in the top 10, 8, 5, and 3 percent of the IP'd fragments were compared to existing RAP1 and SIR data (Lieb, Liu et al. 2001). Features ranking in the top 5 percent or better were considered good RIF1 telomeric targets since they had a high correlation to RAP1 and the Sir2-4 proteins at telomeres. Top-ranking features were compared to the entire yeast genome using BLAST to assess the extent of potential cross-hybridization. Fragments with over 70% identity were considered "redundant" for analysis purposes, while those with less than 70% identity were considered "unique". The distance of DNA association from the chromosome ends for Rif1-proA was expressed as the centromeric coordinate of all the top-ranking positive features that were located within 15kb of the chromosome end. The "innermost distance from the end" measurement (IDE) was determined for each end and compared to the lengths determined for Rap1p and the Sir2-4 proteins using the same analysis method (Lieb, Liu et al. 2001). DNA association maps were plotted using Promoter version 3.25 (Generous gift of Joseph Derisi).

Results

Characterization of Epitope-tagged Strains

In order to examine the ability of telomeric components to immunoprecipitate (IP) telomeric DNA through the cell cycle, we epitope-tagged the *RIF1*, *RIF2*, and *EST2* genes in strains using PCR-based recombination. Each tagged gene retained its endogenous promoter and was present at its normal chromosomal location. The expression of epitope-tagged genes was confirmed by Western blotting analyses after IP. All strains showed the expected band upon Western blotting (data not shown) except for Rif2-proA, which exhibited an unexpected doublet after IP, with one band migrating at the expected size for the tagged product and the other migrating more slowly (see results below). Only epitope-tagged strains with telomere lengths and distributions that were stably wild-type or near wild-type over time were used for further studies. These included the Rif1-proA, Rif1-9xMYC, Rif2-proA, and Est2-13xMYC strains (Figure 1, lanes 1-6). Following α-factor arrest and release, the cell cycle progression of each epitope-tagged strain was monitored by budding indices and compared to WT. In all cases, the WT and tagged strains progressed through the cell cycle with similar budding kinetics after release from α-factor arrest (data not shown). These data suggested that these epitope-tagged strains had WT-like telomeric chromatin complexes.

Chromatin Immunoprecipitation Controls

We first experimentally validated that the IP of telomeric chromatin from our yeast strains was dependent upon both the presence of the appropriate epitope and the

treatment with crosslinking agent. Two types of background controls were performed. For Rap1p and Est2-13xMYC controls, WT strains were "mock IP'd" without a primary antibody, but with protein A- or G sepharose beads. Controls for Rif1-proA and Rif2-proA IP's consisted of the lysates from isogenic untagged (i.e. wild-type) cultures IP'd in the presence of IgG sepharose. The average amount of telomeric DNA IP'd from mock IP's and untagged controls were comparable (0.14% of input, Figure 2). For later statistical analyses, the values obtained using both types of background controls were averaged. On average, telomeric DNA IP'd from wild-type strains using α-Rap1p antibodies was enriched about 5-fold over mock IP's (0.74% of input, Figure 2), while Rif1-proA IP's enriched telomeric DNA about 28-fold over IP's from untagged control strains (4% of input, Figure 2). Rif2-proA IP's enriched telomeric DNA about 3-fold over IP's from untagged control strains (0.42% of input, Figure 2), and Est2-13xMYC IP's enriched telomeric DNA about 2.5-fold over mock IP's from wild-type strains (0.35% of input, Figure 2). The detection of telomeric DNA in ChIPs assays was dependent on crosslinking agent in all cases, although Rap1p and Rif1p did IP a detectable level of telomeric DNA in its absence (Figure 2). With the exception of Rif1-proA and Rif2-proA, it is difficult to directly compare the amount of IP'd telomeric signal between proteins, since different antibodies were used to IP each component. Since both Rif1p and Rif2p were -proA tagged, their signals can be more directly compared: in our assays Rif1-proA was, on average, 10-fold more crosslinkable to telomeric DNA than was Rif2-proA.

RAP1 Association with Telomeric Chromatin Through the Cell Cycle

The crosslinkability of Rap1p to telomeric DNA through the cell cycle was monitored in three independent timecourse experiments. Wild-type BY4736 strains were synchronized by α-factor arrest, released, and allowed to proceed through a complete cell cycle into the subsequent G1 phase. Lysates were made at each timepoint. Half of each lysate was IP'd with α-Rap1p antibodies while the other half was mock IP'd without primary antibodies, as a background control. In the left panels of Figure 3A, the total height of each black histogram bar represents the total amount of IP'd telomeric signal. The level of background signal from control mock IP's is shown on the same scale, overlayed as the white bars. While control mock IP's did not show significant differences in signal over the cell cycle, Rap1p samples did show significant changes in the amount of telomeric DNA IP'd. The change in Rap1p signal was apparent both as the raw percent of input DNA (Figure 3A, left panel black bars) and when individual timepoint signals were normalized to the average IP'd signal to determine the fold change through the cell cycle (Figure 3A, right panel).

Crosslinkability of Rap1p to telomeric DNA was minimal 40-60 minutes after α-factor release, corresponding to early and mid S-phase. Crosslinkability increased rapidly from 60-80 minutes and then decreased during mitosis and through G1 of the next cell cycle. Both the timing and extents of decrease in Rap1p crosslinkability to telomeric DNA are consistent with previous microscopic studies that indicated that half the Rap1p is displaced from telomeres as cells pass through mitosis (Laroche & Gasser 99 EMBO).

RIF1 Association with Telomeric Chromatin Through the Cell Cycle

The ability of Rif1-proA to IP telomeric DNA through the cell cycle was determined. Both the raw IP'd telomeric signals (Figure 3B, left panel black bars) and signals normalized to the average of all timepoints (Figure 3B, right panel) showed similar trends. While the variability of Rif1-proA signal was high at the first and last timepoints after α-factor release (20 and 120 minutes respectively, each timepoint corresponding to G1 in the cell cycle), there were significant and consistent changes in the amount of telomeric DNA precipitated by Rif1-proA over the rest of the cell cycle. Rif1-proA-associated telomeric signal was minimal at the 40- and 100-minute timepoints, corresponding to the beginnings of S-phase and G1 respectively, and was maximal at the 60- and 80-minute timepoints after α-factor release. These timepoints corresponded to mid-late S-phase and G2 in the cell cycle. This increase in the chromatin association of Rif1-proA in late S-phase and G2 is consistent with the increase in *RIF1* gene transcription observed during S-phase (Cho & David 98 Cell). Notably, 60 minutes after α-factor release, Rif1-proA became relatively more crosslinkable to telomeric DNA than Rap1p (Figure 3A & 3B, right panels). This may reflect a stronger association of Rif1-proA with the remaining telomere-bound fraction of Rap1p, or higher accessibility of Rif1-proA on telomeres than Rap1p. Alternatively, the increased association of Rif1-proA with telomeric DNA at 60 minutes may occur through a binding partner other than Rap1p. Like Rap1p, Rif1-proA was displaced between 80 and 100 minutes, which correspond to mitosis and entry into the next cell cycle respectively. The high variability of the Rif1-proA telomeric signal at the 20- and

120-minute timepoints precluded determining if Rif1-proA increases or decreases its crosslinkability to telomeric DNA through G1.

RIF2 Association with Telomeric Chromatin Through the Cell Cycle

The amount of telomeric DNA precipitated by Rif2-proA, relative to untagged controls, changed significantly through the cell cycle (Figure 3C). Notably, the trend over the timecourse for Rif2-proA was distinct from those of both Rap1p and Rif1-proA. The amount of IP'd telomeric DNA progressively decreased from 20-80 minutes after α-factor release, corresponding to progression from G1 to G2. Although the absolute enrichment of Rif2-proA crosslinked telomeric DNA over untagged controls was less than that of Rap1p or Rif1-proA, this decrease through the cell cycle was statistically significant. Rif2-proA slightly increased its association with telomeric DNA through mitosis and into the next cell cycle. Similar changes were observed whether the numbers were analyzed as raw signals (Figure 3C, left panel black bars) or the signals normalized to the average signal for the timecourse (Figure 3C, right panel). When raw Rif2-proA telomeric signal was further corrected for the signal of untagged controls, a similar decreasing trend through the cell cycle was seen; however, this decrease began 40 minutes after α-factor release instead of immediately (Supplementary Data). We conclude that regardless of the signal correction or analysis method used, there is a robust decrease in Rif2-proA telomere crosslinkability through G1, S, and the G2 phases of the cell cycle. This is inverse to the general trend of increased telomeric crosslinkability for Rif1-proA and Rap1p through S-phase and G2 (Compare Figure 3A & 3B to 3C). Similarly, through mitosis and the subsequent G1 phase, the amount of

crosslinkable telomeric DNA appeared to increase for Rif2-proA, while it decreased for Rif1-proA and Rap1p.

EST2 Association with Telomeric Chromatin Through the Cell Cycle

Crosslinkability of Est2-13xMYC to telomeric DNA changed during the cell cycle (Figure 3D). Overall, the telomeric signal IP'd by Est2-13xMYC was weaker than that for Rap1p, Rif1-proA, and Rif2-proA (2.5-fold over mock IP background control, Figure 2). Although the variability of Est2-13xMYC IP'd telomeric signal was relatively high from 20-80 minutes following α-factor release, there was a statistically significant decrease in IP'd telomeric signal at 100 minutes after α-factor release, corresponding to the end of mitosis (Figure 3D, right panel). These data suggest that Est2-13xMYC may remain associated with the telomere throughout much of the cell cycle and became displaced or inaccessible to ChIP as cells pass through mitosis. Interestingly, when Est2-13xMYC crosslinkability over the course of the cell cycle is further corrected for the background of mock IP's, it increases telomeric association at the beginning of S-phase, peaks in late S-phase, and shows a massive decrease through G2 and mitosis (Supplementary Data Figure B). This is consistent with the known time of telomere elongation by telomerase in late S-phase through G2 (Diede and Gottschling 1999; Marcand, Brevet et al. 2000). Recent studies of replication timing have shown that origins within 35 kB of the telomere begin replicating in early-mid S phase and replication of the telomeric regions is largely complete by mid S-phase. Strikingly, however, our data indicate that Est2-13xMYC is associated with telomeric DNA significantly before telomerase acts or telomere replication occurs, and that Est2-

13xMYC largely loses it crosslinkability to telomeric DNA after the time of telomeric replication.

Colocalization of RAP1 with RIF1

As an independent method to assess the physical interaction between Rap1p and Rif1-9xMYC with DNA and one another through the cell cycle, we localized these proteins by immunofluorescence in chromatin spreads (Klein, Laroche et al. 1992; Michaelis, Ciosk et al. 1997). In this method, spheroplasts are gently disrupted and their nuclear contents are spread locally on the slide. Associated proteins are then paraformaldehyde-fixed to the DNA but are dispersed over a larger area than in unspread nuclei, facilitating their visualization. Chromatin spreads were performed using cells of the W303-1a strain background. Comparison of budding indices and FACS profiles from control α-factor arrest/releases showed that untagged and epitope tagged W303a and S288C strains had a similar response to α-factor and similar kinetics of release and progression through the cell cycle (data not shown). Previous evidence has shown that Rap1p colocalizes with Y' elements at the telomeres in relatively intact nuclei (Gotta, Laroche et al. 1996) and that Rif1p and Rap1p are colocalized *in vivo* (Mishra and Shore 1999). For each cell cycle timepoint, the total number of Rap1p- and Rif1-9xMYC-staining foci per spread nucleus was counted for about 75 DAPI staining areas (i.e. nuclei) (Figure 4A). The average number of staining foci per spread nucleus was then calculated for each protein and timepoint. We observed between 4 and 9 (average of 6.4) Rap1p foci per spread nucleus through the cell cycle (Figure 4B, black line). This is similar to the reported number of Rap1p foci visible in intact cells

(Laroche, Martin et al. 2000). We observed between 4 and 6 Rif1-9xMYC foci (average of 4.9) per spread nucleus through the cell cycle (Figure 4B, dashed line). The number of visible, discrete Rap1p and Rif1-9xMYC foci was highest 40 minutes after α-factor release, in early S-phase, and lowest at 60-80 minutes after α-factor release, corresponding to late S-phase and G2 (Figure 4B). Overall, the trends in the number of foci for Rap1p and Rif1-9xMYC over the cell cycle were well correlated. As the number of Rif1-9xMYC spots decreased in number they also tended to increase in size. When the number of Rif1-9xMYC spots per spread nucleus was lowest (i.e. 60-80 minutes after α-factor release), the percentage of spread nuclei with a few, relatively large, Rif1-9xMYC spots was maximal (Figure 4A, white arrowheads). While the number of Rap1p spots also decreased through the cell cycle, the spots did not appear to "coalesce" in the same manner as Rif1-9xMYC.

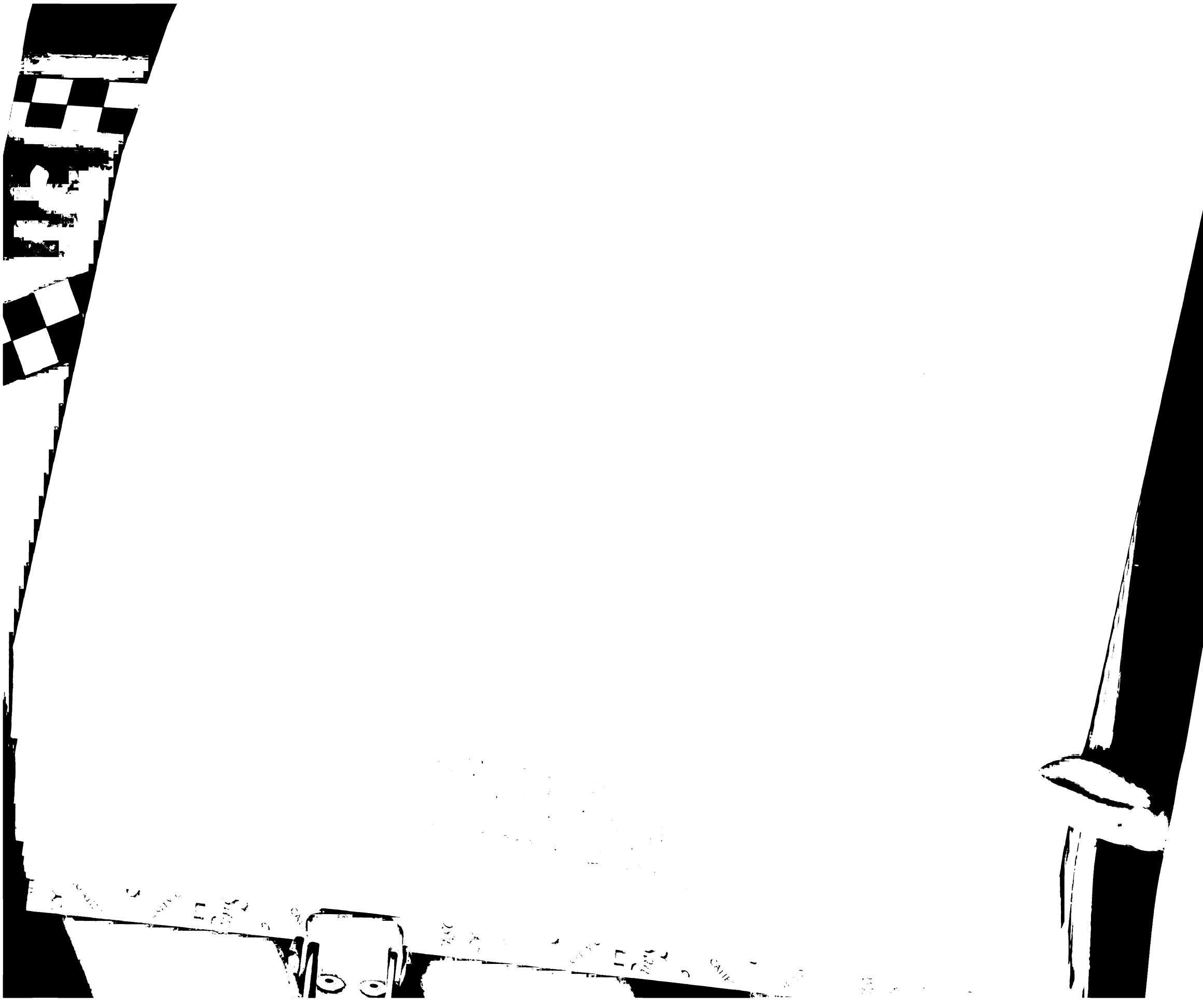
Next, we assessed the extent of colocalization of Rap1p and Rif1-9xMYC through the cell cycle. In G1 and early S-phase, when more spots were present, there was a low percentage of colocalization (Figure 4A and 4C), and as the number of spots decreased, the "compaction" and the percentage of Rif1-9xMYC colocalization increased (Figure 4A and 4C). Thus, when there were the fewest Rif1-9xMYC spots (i.e. 60 minutes after α-factor release) there was the highest percentage of the remaining Rif1-9xMYC spots colocalizing with Rap1p spots. Strikingly, from mid-S phase to G2, when the number of Rap1p and Rif-9xMYC foci was lowest, their ability to IP telomeric DNA was greatest in the ChIP assays (Figure 3A & 3B). This suggests that the few, clustered spots remaining were associated with telomeric DNA.

Determination of Genome-wide RIF1 Targets by Microarray Analysis

We further investigated association of RIF1-proA with chromatin using microarrays that contained all *S. cerevisiae* ORFs and intergenic regions (Gerton, DeRisi et al. 2000; Iyer, Horak et al. 2001; Lieb, Liu et al. 2001). We determined which telomeres were bound by Rif1-proA, how far in from the chromosome ends it associated, and whether Rif1-proA has non-telomeric binding sites. We used median rank analysis to determine which genome features or targets (i.e. ORFs and intergenic regions) were consistently enriched in at least two of three independent array experiments using DNA precipitated from asynchronous Rif1-proA cultures in mid-log phase (Iyer, Horak et al. 2001; Lieb, Liu et al. 2001). Briefly, in this ranking method, those DNA targets are sorted in order of their red to green ration and features that are consistently enriched by the ChIP procedure show a higher median percentile rank. In previous experiments examining the genome-wide DNA association of chromatin factors, two general trends in the data have been observed: proteins that bind DNA directly appear to have a bimodal distribution of targets, with the highest-ranking targets forming a small peak at the edge of the main, normal distribution. An example of this is seen with Rap1p, which shows that the top 8 percent of the distribution is enriched for Rap1p binding (Lieb, Liu et al. 2001). In contrast, for factors that do not bind DNA directly, the distribution of features appears as a roughly normal, rather than a clear bimodal, distribution. In these cases it is not possible to unambiguously assign a threshold above which enriched features are deemed "significant binding targets". In some cases, as with the G1/S-phase transcription factor MBF (MBP/Swi6 heterodimer) (Iyer, Horak et al. 2001), it is possible to correlate enriched ChIP features with mRNA

expression. Rif1-proA, crosslinkability to genomic DNA appeared as a normal distribution, consistent with its known indirect association with DNA through Rap1p (Hardy, Sussel et al. 1992). We analyzed the top 10-, 8-, 5- and 3 percent of enriched ChIP targets and empirically determined that the top 5 percent of features from the distribution were consistently enriched for telomeric targets. Furthermore, Rif1-proA binding sites in telomeric regions correlated with regions bound by Rap1p, Sir2p, Sir3p, and Sir4p (Figure 5) (Lieb, Liu et al. 2001).

A total of 325 genomic features ranked in the top 5 percent of Rif1-proA IP'd fragments in at least two of three experiments. A total of 84 of these, all located within 15 kB of the chromosome ends, were generally highly enriched in the Rif1-proA ChIPs. Thus, while the last 15 kB of all 32 telomeric regions represents only 4% of the total genomic sequence, 26% of the enriched genomic features were in these regions. We next compared the 325 Rif1-proA targets with the top-ranking 8% of Rap1p targets (Lieb, Liu et al. 2001). Rap1p and Rif1-proA shared only 85 targets. Strikingly, 75 of these, or 88%, were within 15 kB of the chromosome ends. This result suggests that Rap1p and Rif1-proA are highly correlated specifically at chromosome ends. Of the remaining 10 non-telomeric sites in common for these proteins, 4 were are the HMRA2/MATA2 locus. The remainder showed no coherent pattern (COX11, IMD3, SEC10, RNC1 SES1 and MRL1). In summary, while Rap1p and Rif1-proA are highly correlated at chromosome ends, they do not appear to have generally similar internal genomic targets other than HMR. This is consistent with previous observations that Rif1p, unlike Rap1p, is non-essential, and apparently is not required for any critical transcriptional activation functions mediated by Rap1p (Hardy, Sussel et al. 1992).



We measured the extent of Rif1-proA association to individual chromosome end regions using an analysis similar to that described for Rap1p and the Sir2-4 proteins (Lieb, Liu et al. 2001). We chose 15 kB as the furthest distance from the end where we would begin to consider positive RIF1 targets, since this was the interval where Rap1p and the Sir2-4 proteins were the most colocalized (Lieb, Liu et al. 2001). We then took the centromere-proximal coordinate for those Rif1-proA targets and defined this value as the "innermost distance from the end", or IDE, to which Rif1-proA bound. The IDE-measurement was repeated for all 32 chromosome ends (Supplemental Data). Using data from all detected chromosome ends, we determined that the IDE measurement for Rif1-proA averaged 6.4 kB in from the ends, with a minimum value of 0.47 kB and a maximum of 13 kB. It is important to note that the IDE-measurement does not imply that Rif1-proA is continuously associated from this point to the chromosome end. Like Rap1p and the Sir2-4 proteins, Rif1-proA was generally found to be associated with a number of fragments at each chromosome end (Figure 5).

One complication in the analysis of telomeres from microarray data is that many chromosome ends are highly homologous to one another. Thus, Rif1-proA targets that have many homologous sequences within the genome are overrepresented. For example, in *S. cerevisiae*, 17 out of the 32 chromosome ends contain the repetitive, highly homologous Y' elements. This redundancy of subtelomeric DNA may lead to overestimation of the extent of Rif1-proA binding from the chromosome end. In order to address this issue we examined the Rif1-proA IDE measurements using only targets with a homology to the rest of the genome of less than 70%. Using this "unique" target data set of 15/84 top-ranking features within the last 15kB, we determined that the IDE

for Rif1-proA was 5.3 kB (Supplemental Data). Conversely, when we used only "redundant" targets, whose homology was greater than 70%, to estimate the Rif1-proA IDE, the result was 6.6 kB (Supplemental Data). This range of Rif1-proA values did not differ significantly from those of Rap1p or the Sir2, Sir3, and Sir4 proteins, which were 6.8, 7.0, 6.3, and 6.6 kB respectively (Supplemental data). These analyses indicate that the IDE to which Rif1-proA and the Sir2-4 proteins can associate is quite similar for each chromosome end, suggesting that the Rif1 and Sir2-4 proteins may not be strictly partitioned between the telomere and subtelomere as previously suggested (Wotton and Shore 1997), but may instead simultaneously occupy these regions.

We were interested if there were any significant differences between IDE-measurements on chromosome ends that contained X-elements versus Y' elements. Using the information gathered from comparing the identity of chromosome ends to one another and existing annotations (http://www.le.ac.uk/genetics/ejl12/EndsData.html), we separated our IDE-measurements into X-element and Y' element classes. We then compared the IDE-measurements for Rif1-proA, Rap1p, and the Sir2-4 proteins for these two types of chromosome ends (Figure 5). Strikingly, there was a highly significant ($p \leq 0.003$) length difference based on the type of end. For X-element ends, the average Rif1-proA IDE-measurement was 3.7 kB using data from all ends, 3.6 kB using unique target data, and 3.3 kB using only the data from redundantly detected targets (Figure 5, Supplemental Data). For telomeres with Y' elements, the Rif1-proA IDE-measurement was 8.1 kB using all end data, 7.5 kB for unique target data, and 8.9 kB for redundantly detected targets (Figure 5, Supplemental Data). There was not a significant difference in the IDE-measurements for chromosomes with Y'-long (6.7 kB)

versus Y'-short (5.2 kB) elements (data not shown). The difference between IDE-measurements between X-element and Y' element chromosome ends for Rap1p and the Sir2-4 proteins was highly correlated with Rif1-proA and ($r>0.98$) and similarly significant ($p\leq0.003$). Thus, it appears that on average, telomeric proteins associate twice as far in from the chromosome ends on telomeres with Y'-elements than on those with only X-elements. Y' elements are bounded at both ends by short, ~150 bp, tracts of telomeric repeats. These internal tracts are shorter than the terminal telomeric tracts, but because they flank each of the 5.2 and 6.7 kB Y' elements, they can be many kilobases in from the chromosome ends. These data suggest that the internal $TG_{1\text{-}3}$ tracts that flank Y' elements are capable of recruiting telomeric components and may contribute to overall telomere chromatin structure.

A total of 239 targets associated with Rif1-proA were not associated with Rap1p (Supplemental Data). While it is difficult to discern how these Rif1p targets are inter-related, of particular interest were a number of genes involved in various aspects of telomere maintenance. The *TEL1*, *Ku70*, *POL1*, *SME1*, *MLP1*, and *MEC1* genes, all involved in telomere maintenance, were found amongst the top 3% of non-telomeric targets. The *TEL1* and *MEC1* genes are thought to be involved in sensing telomere length or signal transduction at the telomere. *The SME1* gene is an SM-family protein and is involved in processing the telomerase RNA gene (*TLC1*) (Seto, Zaug et al. 1999). The *MLP1* gene encodes a nuclear pore component that interacts with *MLP2*. The *MLP2* gene product interacts with the Ku proteins and provides a possible link between the telomeric chromatin and nuclear periphery (Galy, Olivo-Marin et al. 2000). The likelihood of randomly enriching 6 genes involved in telomere biology in ChIPs is

highly unlikely ($p<8x10^{-13}$). It will be of interest to determine whether RIF1 regulates these genes.

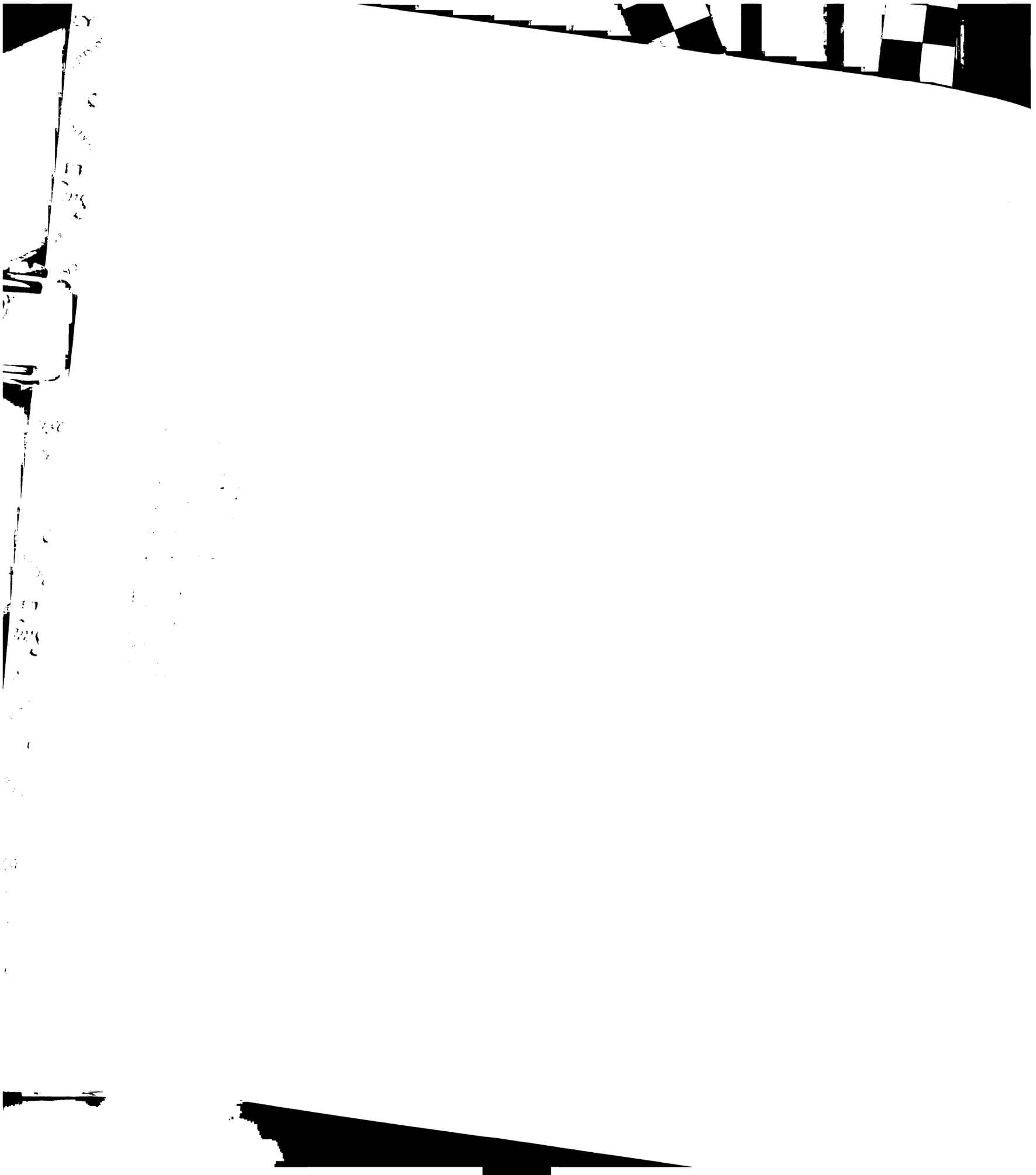
A Cell-Cycle Dependent Modification of Rif2p

Rif2-proA exhibited two reactive species in Western blot analyses of IPs. One band migrated at approximately 58 kD, the expected size for Rif2-proA, and the other at ~70 kD (Figure 6A and 6B). In HA- or TAP- tagged Rif2p strains, the apparent difference in size for the doublet bands was ~5 kD (D.L. Levy, C.D. Smith & E.H. Blackburn unpublished data). When Rif2-proA epitope-tagged strains were synchronized with α-factor, released into the cell cycle, IP'd with IgG sepharose, and analyzed by Western blotting using α-Rif2 antibodies, the ratio of the two doublet bands changed over the cell cycle. As shown in Figure 6A, at 20 minutes after release (G1), the ratio was 1:1 in Western blotting analysis. The ratio of the upper 70 kD to the lower 58 kD species increased dramatically from 40-80 minutes (S-G2), returned to 1:1 at 100 minutes (G1 of the subsequent cell cycle), and increased again at 120 minutes (early-mid S-phase). Thus, in G1 Rif2p appeared to be equally in the 58 kD and 70 kD form, while in S-phase it appeared to be mainly in the modified, 70 kD form.

We investigated some potential modifications that might be responsible for the altered mobility of Rif2-proA. One protein modification, known as SUMOylation, is consistent with the observed size difference between the two Rif2p bands. SUMO, a 12 kD ubiquitin-like protein that is encoded by the *S. cerevisiae SMT3* gene (Schwarz, Matuschewski et al. 1998, Johnson, 1997 #69) is important for proper chromosome segregation in budding yeast (Biggins, Bhalla et al. 2001) and requires the E2-like

Ubc9p activity to conjugate it to proteins (Johnson, Schwienhorst et al. 1997; Schwarz, Matuschewski et al. 1998). Using the *ubc9-1* temperature sensitive allele of UBC9, which is not competent for conjugation of Smt3p to proteins at the non-permissive temperature, we found no change in the Rif2-proA doublet (Figure 6B, lane 4). Furthermore, co-IP of Rif2-proA in a strain containing a GST-tagged version of the SMT3 gene did not yield a cross-reactive Rif2-proA-GST-SMT3 band (data not shown). Western blots with Rif2-proA doublets were also stripped and re-probed with 3 different antibodies to ubiquitin. There were no cross-reactive Rif2-proA species detected with α-ubiquitin antibodies (data not shown). Hence, neither SUMOylation nor mono-ubiquitination appears to account for the Rif2-proA doublet.

Other factors potentially involved in the Rif2-proA modification were also tested. Recent evidence suggested an interaction between the NAD-dependent deacetylase Sir2p and Rif2p (Ito, Chiba et al. 2001). Other experiments have shown that the *TEL1* And *MEC1* genes are genetically epistatic to the *RIF* genes (Craven and Petes 1999; Chan, Chang et al. 2001). We generated Rif2-proA-tagged strains containing a *SIR2* deletion strain, a triply deficiency of *TEL1 MEC1 SML1*, or a *RIF1* deletion, but observed difference in the Rif2-proA doublet, from wild-type. Thus, neither ATM kinases nor Sir2p are required for the putative Rif2-proA modification (Figure 6B, lanes 3 and 5). Likewise, Rif2p modification is not changed when telomeric the chromatin is disrupted by deletion of the *RIF1* gene (Figure 6B, lane 2). To address whether redundant or other kinases might be responsible for the observed doublet, Rif2-proA lysates were prepared in the presence of the phosphatase inhibitors NaF, Na_3VO_4, and Na-β-galactosidase, or treated with λ-phosphatase (NEB, Beverly MA). Neither the

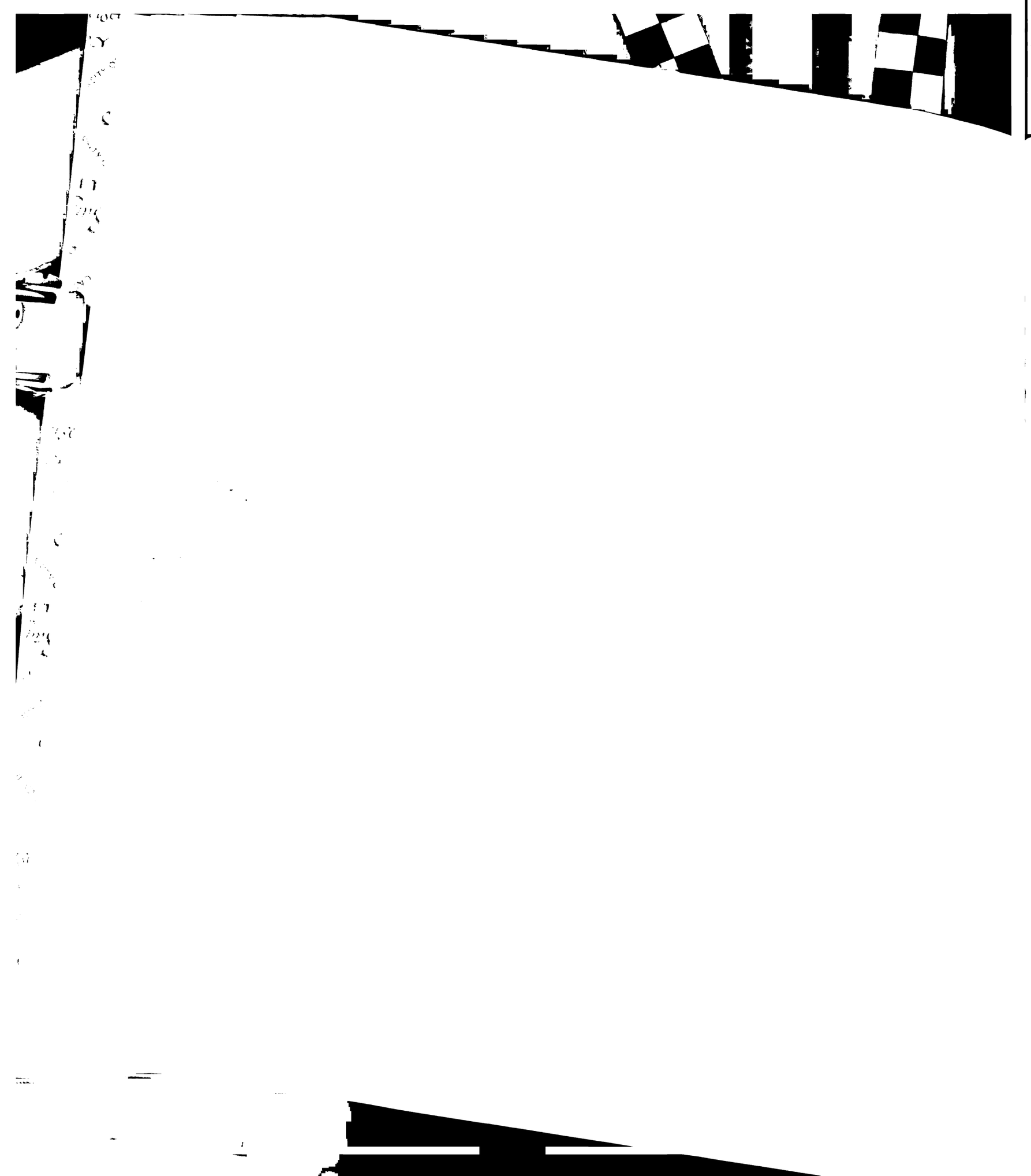


presence of inhibitors nor phosphatase treatment affected the observed Rif2-proA doublet, suggesting that phosphorylation is not responsible for the apparent mobility shift (data not shown).

Association of Rif1p and Rif2p is Relatively Increased on Uncapped Telomeres

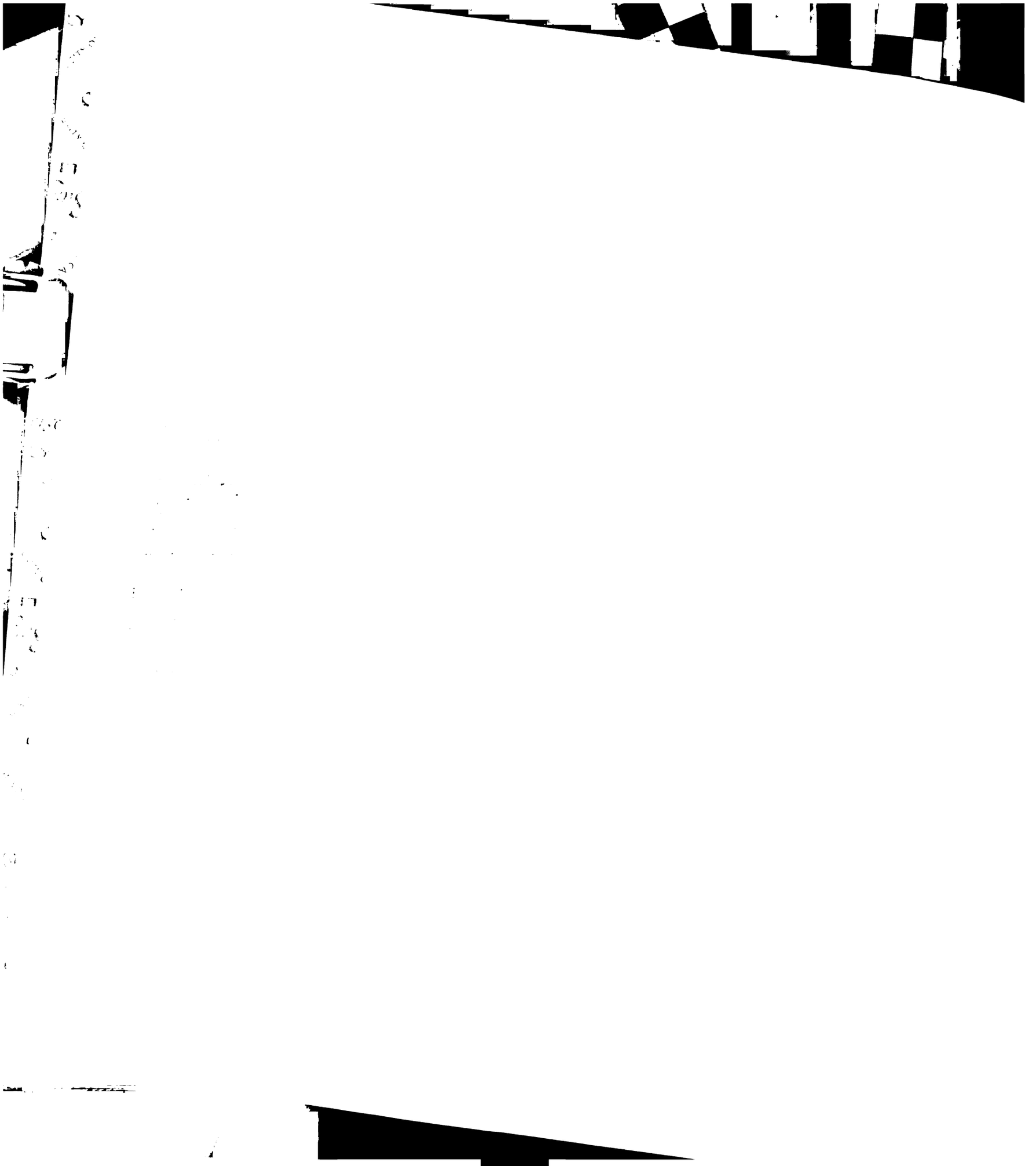
To gain more insight into the regulation of the chromatin association of Rap1p, Rif1-proA, and Rif2-proA, we investigated two situations in which the ability to strictly regulate telomere length homeostasis was compromised. First, we examined heteroallelic *tlc1-476A/TLC1* strains. The *tlc1-476A* allele contains a C to A transversion in the core binding site for Rap1p (Chan, Chang et al. 2001). While this mutation allows both *in vitro* and *in vivo* telomerase activity, strains homozygous for this mutation have elongated, deregulated telomeres containing aberrantly long $(TG)_n$ tracts of up to 60bp. Telomeres in *tlc1-476A* strains also undergo extensive degradation, and have significant growth defects. Heteroallelic *tlc1-476A/TLC1* strains, on the other hand, have no detectable cell growth defects, but do undergo telomere lengthening, lose some of their ability to regulate length, and exhibit mild telomeric DNA degradation (Figure 1, lanes 8, 11, 12). Also, heteroallelic *tlc1-476A/TLC1* strains have normal chromosome segregation, while homozygous *tlc1-476A* strains show significant chromosome missegregation (D.L. Smith & E.H. Blackburn unpublished data). Therefore, telomeres in *tlc1-476A/TLC1* strains are largely WT functionally, despite their increased tract length and loss of length regulation.

We generated heteroallelic *tlc1-476A/TLC1* strains containing the *tlc1-476A* template mutation integrated next to a WT copy of the telomerase RNA gene, *TLC1*. We assessed the quantitative ability of Rap1p, Rif1-proA, and Rif2-proA to IP telomeric DNA in these cells. As expected, their terminal tract lengths of telomeres were significantly longer than WT (Figure 1A, lanes 8, 11, 12). Correspondingly, the input telomeric signal,



as detected by Southern Blot and dot blotting analyses using a WT telomeric probe, was 2-5 fold greater in *tlc1-476A/TLC1* strains than WT (data not shown). The percentage of input telomeric DNA that could be IP'd by α-Rap1p antibodies in *tlc1-476A/TLC1* strains was 66% of that in the control WT strain (Figure 7). This decrease could reflect the presence of $(GT)_n$ tracts lacking Rap1p consensus binding sequences. In contrast, the percentage of input telomeric DNA that could be IP'd by Rif1- and Rif2-proA was not significantly decreased in the *tlc1-476A/TLC1* cells compared to WT cells (Figure 7). Thus, despite the increase in total telomeric DNA in *tlc1-476A/TLC1* cells, the absolute amount of associated Rap1p decreased, while the absolute amounts of both Rif1- and Rif2-proA increased.

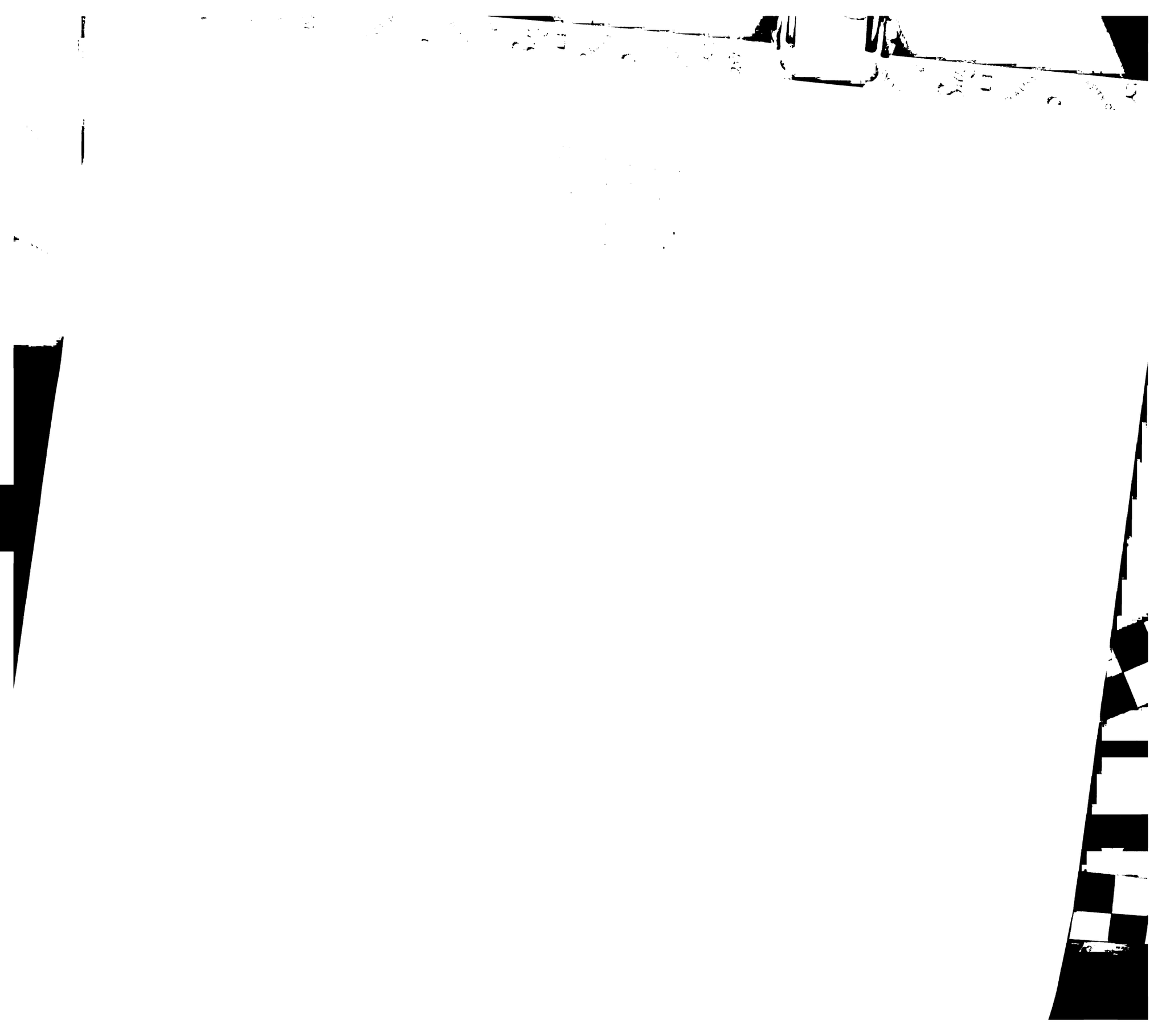
We also examined the relative crosslinkability of Rif2-proA in another situation in which telomere length control is compromised, a *Δrif1* strain. As shown in Figure 1 (lane 13), telomeres in these cells and *tlc1-476A/TLC1* strains were lengthened similarly. As in the *tlc1-476A/TLC1* heteroallelic strains, the relative association of Rif2-proA with telomeric DNA was significantly higher in *Δrif1* than in the WT control (Figure 7). These data suggest that association of Rif2-proA with telomeric DNA increases in two different situations where telomeres are elongated and length regulation is compromised. Previous studies have shown that Rif2p is particularly important in preventing the terminal telomeric tract from participating in the *RAD52*-dependent Type II survivor pathway (Teng, Chang et al. 2000) and is also important in directly inhibiting telomerase addition to *de novo* ends (Diede and Gottschling 1999). Therefore, increased recruitment of Rif1p and Rif2p in *tlc1-476A/TLC1* cells and Rif2p in *RIF1* deletion strains may be a response to the partial uncapping effects of these mutations, possibly as a mechanism to regain length control.



Discussion

Our data indicate that regulated changes in the association properties of telomeric proteins remodel chromatin at the chromosome ends over the cell cycle. Previous immunofluorescence data in yeast suggested that Rap1p, Sir3p and Sir4p are displaced from telomeres in late G2/M (Laroche, Martin et al. 2000). Association of the human Xrs2p homolog, NBS1, with telomeres has also been observed specifically in S-phase (Wu, Lee et al. 2000; Zhu, Kuster et al. 2000). In yeast, while *in vivo* telomeric DNA addition occurs in late S-phase and G2/M (Diede and Gottschling 1999; Marcand, Brevet et al. 2000), it had not been determined when in the cell cycle telomerase is associated with, or gains access to, its telomeric substrate. We found that a measurable amount of the core telomerase protein Est2p is associated with the chromosome end through most of the cell cycle, only being displaced in mitosis. Furthermore, we found that Rap1p and Rif1p are maximally associated with telomeres in late S-phase and G2, while Rif2p steadily decreases its telomeric association through S-phase, coincident with its modification. We also demonstrated that Rif1p in the telomeric regions associates to similar extents in from the chromosome ends as the Rap1 and Sir2-3 proteins. Our results suggest that Rif1p, like Rap1p and the Sir2-4 proteins, is capable of associating with the telomeric DNA that flanks Y' elements.

When considered together, the ChIP data for Rap1p and the tagged Rif1, Rif2, and Est2 proteins suggest a finely tuned, dynamic interplay between the telomeric components at the chromosome ends. Notably, there was generally no greater than a 2.5 fold change in the crosslinkability of the telomeric factors investigated in this study. This may reflect the fact that telomere homeostasis is in a dynamic equilibrium of

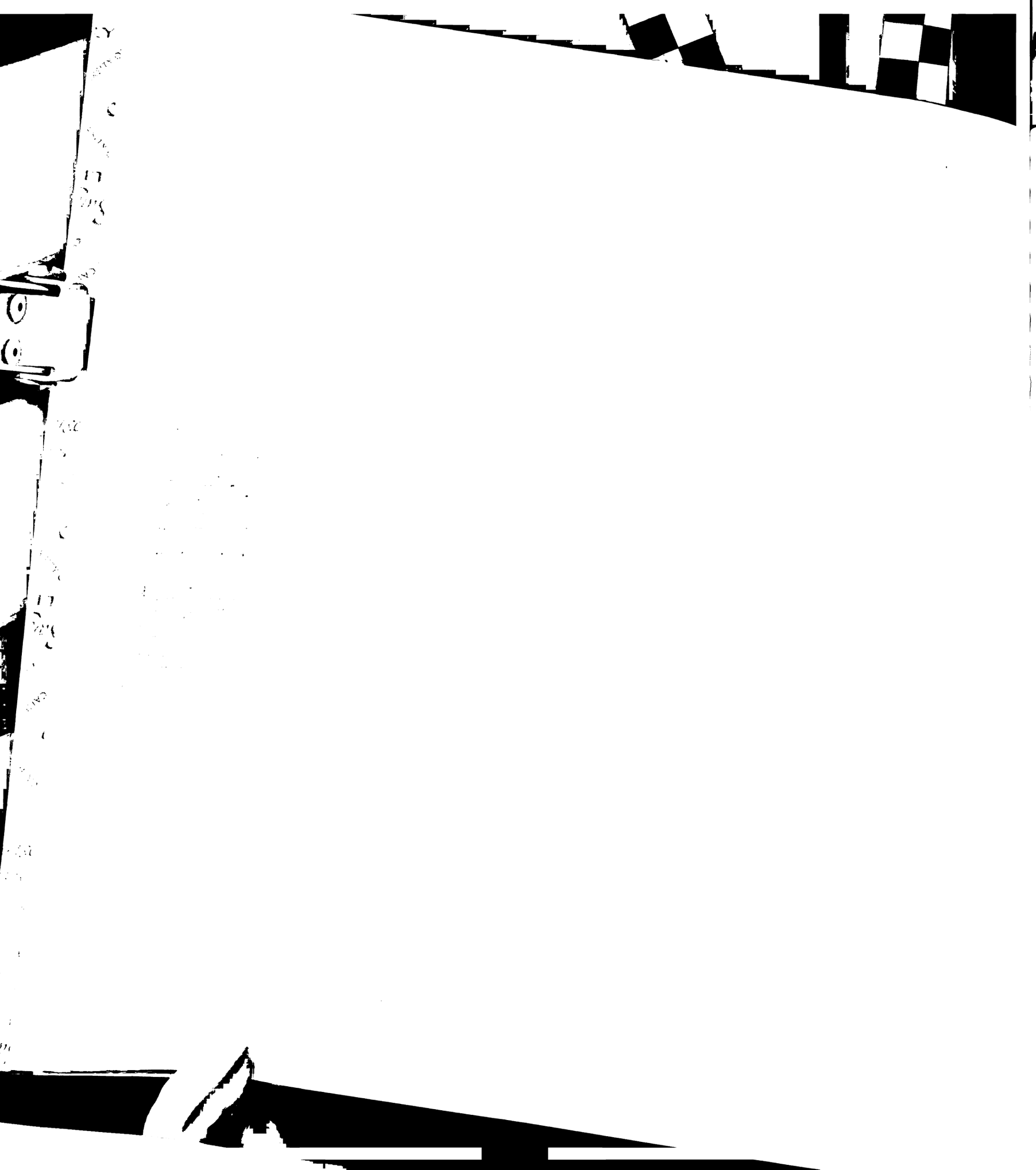


lengthening and shortening activities and that large changes in the chromatin are not required to tip the balance toward telomere lengthening or end protection.

Telomerase and the Cell Cycle

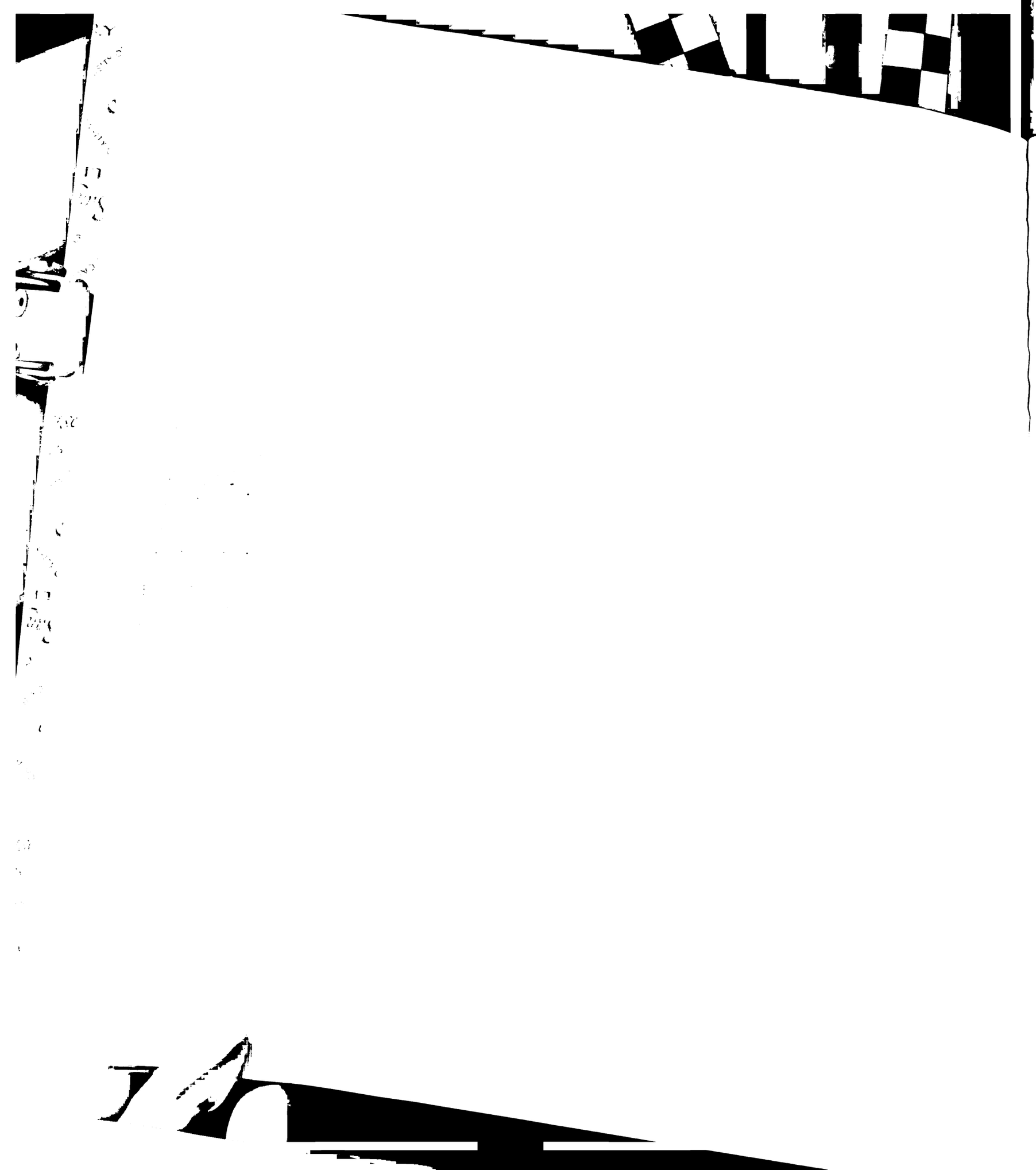
We have shown that Est2-13xMYC is capable of crosslinking to telomeric DNA through most of the cell cycle except mitosis, when its crosslinkability to telomeric DNA was at levels only slightly above background (Figure 3D right panel). Recent experiments suggest that the origins used to replicate telomeric regions may be activated in mid S-phase (Raghuraman, Winzeler et al. 2001), earlier in S-phase than previously thought. Our finding of association of Est2-13xMYC with telomeric DNA throughout S-phase is consistent with these results. Crosslinkability of Est2p decreased as cells passed through mitosis, either because telomerase is displaced from telomeres or, alternatively, becomes inaccessible to antibodies during mitosis. Telomeric DNA, like repetitive rDNA sequences, may be highly condensed (Guacci, Hogan et al. 1994) during mitosis. However, Est2-13xMYC crosslinkability to telomeric DNA was detectable at points in the cell cycle when Rap1p and Rif1-proA association were both high and low (compare Figure 3D, 20-80 minutes to Figure 3A & 3B, 20-80 minutes), suggesting that the Est2-13xMYC ChIP signals reflect association to telomeric DNA, rather than differential access of antibodies to the 13xMYC epitope.

Interestingly, telomerase was crosslinkable to telomeric DNA in G1 phase, yet in G1 telomerase neither extends *de novo*-cut ends (Diede and Gottschling 1999), nor acts on intact, shortened telomeres *in vivo* (Marcand, Brevet et al. 2000). One interpretation of the association of Est2-13xMYC with telomeric DNA in G1 is that Est2p loads early in the cell cycle in G1 or early S-phase. This suggests that telomerase may require co-



factors in order to begin to elongate telomeres in late S-phase to G2. These could include Est1p, Est3p, Cdc13p, or replication factors, which are all known to be required for telomerase action *in vivo* (Lundblad and Szostak 1989; Lendvay, Morris et al. 1996; Diede and Gottschling 1999) (Figure 8). Indeed, if the telomeric association of Est2p is judged from ChIP data that has been corrected for the background telomeric signal in mock-IPs, then telomerase also appears to significantly increase its association up to late S-phase (Supplementary Data). This pattern of association is consistent with both the structure of the telomeric substrate in S-phase and the properties of the telomerase co-factors Est1p and Cdc13p. Single-stranded, G-rich tails are detectable specifically in S-phase and provide binding sites for Cdc13p (Wellinger, Wolf et al. 1993). Cdc13p, in turn, interacts with Est1p, which further recruits the core subunits of telomerase, Est2p and *TLC1* (Evans and Lundblad 1999). Transcription of the EST1 gene has been shown to increase in mid-G1 phase of the cell cycle (Spellman, Sherlock et al. 1998), suggesting that more of this protein may be expressed and available later in S-phase to increase telomerase recruitment. This raises the possibility that Est1p may be a limiting cofactor in the telomerase complex. Also, interaction of Cdc13p with the replication protein Pol1p (Qi and Zakian 2000) may help to coordinate telomere addition with late replication. Alternatively, coordination of telomerase activity with the replication machinery may not be possible until later in the cell cycle, when replication forks have been initiated and progressed toward telomerase at the chromosome ends (Figure 8).

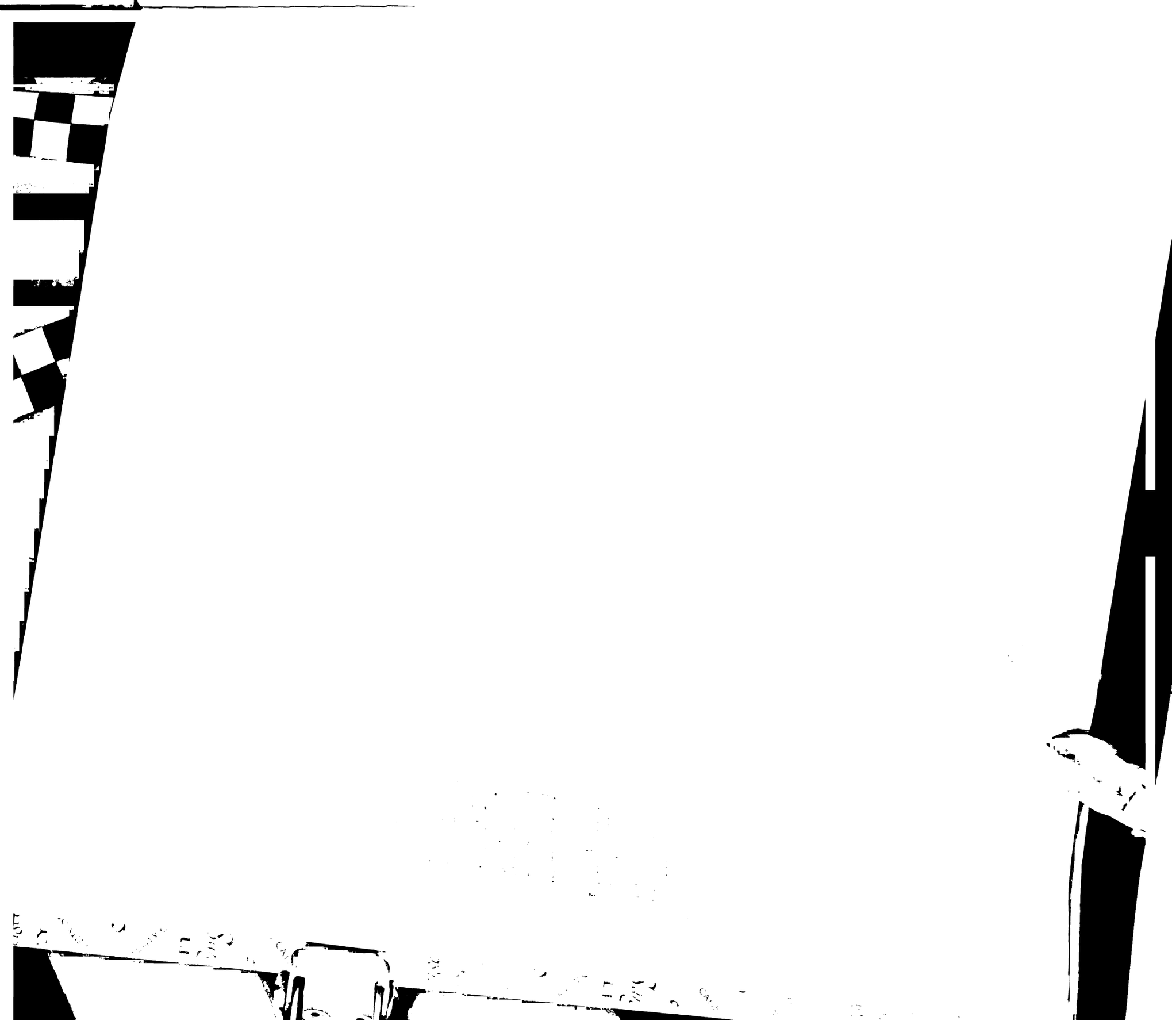
One intriguing possibility raised by either the constitutive or increased association of telomerase with telomeres is that telomeric chromatin may act to sequester telomerase and its cofactors at the chromosome end through the cell cycle.



This may prevent accidental *de novo* synthesis onto inappropriate DNA substrates, such as double strand breaks. Thus, in the same way that repressive telomeric chromatin is thought to act as a "reservoir" of silencing factors used for rDNA silencing (Kennedy, Gotta et al. 1997; Smith, Brachmann et al. 1998), silent mating type loci (Buck and Shore 1995; Marcand, Buck et al. 1996), and DNA damage (Martin, Laroche et al. 1999), perhaps telomeric sequestration of telomerase is a way to prevent potentially promiscuous and detrimental chromosome "healing" events (Jager and Philippsen 1989).

A Model for Cell Cycle Regulation of Telomeric Chromatin

We propose a simple model for the regulation of telomeric chromatin in the cell cycle. This model assumes that the ability to IP telomeric DNA primarily reflects the association of telomeric protein components with the telomeric chromatin. In this model, telomerase associates with the chromosome end through G1, S, and the early G2 phases of the cell cycle and is displaced at G2/M after the last telomeres are fully replicated (Figure 8). The repressive chromatin complex, comprised minimally of Rap1p, Rif1p, and Rif2p, is also dynamically remodeled over the course of the cell cycle. We suggest that the modification of Rif2p through S-phase that we observed correlated with displacement of Rif2p from telomeric DNA and is responsible for progressively opening the telomeric chromatin structure. This may relieve the inhibition of telomerase and its cofactors, thus allowing its action by mid to late S-phase (Figure 8). Perhaps the opening of telomeric chromatin also involves breaking apart the interactions between Rap1p, Rif1p, and the Sir2-4 proteins spread along internal Y' elements and those at the telomere tracts. In our model, as replication forks move



through the end regions and telomeres are replicated, Rap1p and Rif1p quickly reassociate to protect the ends and inhibit over-elongation by telomerase. Once telomere replication is complete, by the end of G2, Rif1p is strongly displaced from the chromatin and unmodified Rif2p re-associates with the remaining Rap1p on the chromatin, thereby inhibiting further telomerase action or recombination (Figure 8). Cells enter the next cell cycle bound by intermediate levels of Rap1p, high levels of Rif2p and eventually telomerase. In this model, the modification of Rif2p modulates its association to the telomere and thereby controls the action of telomerase.

Several lines of evidence support such a model for telomeric chromatin remodeling. Rap1p and Rif1p co-immunoprecipitation with telomeric DNA was highest in G2, when chromosome condensation and packaging is greatest (Guacci, Hogan et al. 1994). Similarly, the time of maximal Rap1p telomere association seen in these ChIP analyses coincided with the fewest positive staining foci seen in chromatin spread assays (late S-G2). Conversely, when there are greater numbers of Rap1p foci (G1 - S-phase), the need to package and cluster telomeres would be least. The genome is actively transcribed and replicated in G1 and S-phase, and the requirement of Rap1p for the transcriptional control of several genes involved in translation and carbohydrate metabolism (Shore 1994; Lieb, Liu et al. 2001) would be expected to be greatest in these phases of the cell cycle. In further support of our association data for Rap1p, the Y'-element helicase is transcriptionally upregulated in the M/G1 phase of the cell cycle (Spellman & Futcher MCB 98), suggesting that these subtelomeric ORFs are accessible to transcription factors and not packaged in repressive telomeric chromatin at this time. These results suggest that fewer, clustered Rap1p foci are correlated with increased

telomeric association and condensation, while more numerous foci are correlated with less condensed telomeric chromatin.

Rif1-proA had a pattern of telomeric chromatin association in ChIP analyses that was similar to, but distinct from, that of Rap1p. The number of Rif1-9xMYC foci observed through the cell cycle in chromatin spread assays also closely mirrored that of Rap1p and, as with Rap1p, as the number of Rif1-proA foci decreased, its chromatin association in ChIP assays increased. A notable difference between Rif1p and Rap1p was the association of Rif1-proA to telomeric DNA earlier in the cell cycle than Rap1p. It is possible that the remaining fraction of Rap1p associated with telomeres in late S-phase may recruit Rif1-proA strongly, giving the appearance that Rif1-proA association precedes that of the bulk of Rap1p. While it might be expected that Rif1-proA chromatin association with telomeric DNA is totally dependent upon Rap1p DNA binding, it is unknown whether other protein-protein interactions might recruit Rif1-proA to DNA. Indeed, microarray data for Rif-1proA suggests that association to non-telomeric genomic targets is independent of Rap1p (Supplementary Data). However, other than Rap1p and Rif2p, no other candidates have been identified that interact with Rif1p in high-throughput 2-hybrid screens (Uetz and Hughes 2000; Ito, Chiba et al. 2001).

While the telomeric association of Rap1p and Rif1-proA increased dramatically through S-phase until G2/M, Rif2-proA steadily decreased its association to telomeres through the cell cycle. These changes in Rif2-proA association were strikingly correlated with a cell cycle dependent change in a Rif2-proA doublet. While the biochemical basis for this doublet is currently unknown, it is notable that the build-up of

the 70 kD Rif2-proA species is coincident with the times of maximal Rif2-proA displacement from the chromosome ends during S-phase and G2 in ChIP assays. Rif2p and Rif1p associate *in vitro* (Wotton and Shore 1997). Therefore, we propose that Rif2p modification blocks its interactions with Rif1p or Rap1p, destabilizing telomeric chromatin to make it more accessible to telomerase and its cofactors.

Genetic evidence suggests that Rif1p and Rif2p act in distinct pathways to maintain telomeres, since deletion of both genes results in synergistic loss of length control (Wotton and Shore 1997). Our model could explain this observed synergism. The known times in the cell cycle of *in vivo* telomeric DNA addition, late S-phase and G2, coincide with the times in the cell cycle where we found Rif2p modification was greatest and its association with telomeric DNA was lowest. In *Δrif2* strains, telomeres elongate slightly and remain regulated. One possibility is that Rif2p plays a greater role in inhibiting telomerase and recombination activities (Diede and Gottschling 1999; Teng, Chang et al. 2000), while Rif1p plays a more structural role with Rap1p at the chromosome ends. Rif1-proA is considerably larger than the Rif2 protein and appears to associate 10 times more strongly with DNA than Rif2-proA in ChIP assays (Figure 2). Rif1p also appears to be spread over many kB of DNA at the end regions, much like Rap1p and the Sir2-4 proteins (Strahl-Bolsinger, Hecht et al. 1997; Lieb, Liu et al. 2001), while Rif2p shows a more restricted association to the extreme chromosome ends in microarray experiments (data not shown). We propose that Rif2p directly inhibits telomerase at the telomeres. Perhaps in the *Δrif2* background telomerase might be freed of this inhibition by Rif2p and therefore able to add telomeric DNA earlier in the cell cycle, resulting in slightly longer telomeres. In this situation, the repressive structural

effects of Rap1p and Rif1p are still present and capable of preventing runaway telomere lengthening or deregulation. In a *Δrif1* strain, telomere lengthening is more substantial, and interaction between Rap1p and Rif2p is increased (Wotton and Shore 1997). Similarly, in our experiments, the deletion of the *RIF1* gene caused increased association of Rid2p with telomeric DNA (Figure 7). The telomere lengthening observed in *Δrif1* strains (Hardy, Sussel et al. 1992; Wotton and Shore 1997)(Figure 1, lane 14,15) and our ChIP data suggest that this increased Rif2p association alone to telomeric DNA is not sufficient to reestablish a repressive chromatin structure and inhibit telomerase (Figure 1, lane and Figure 7). Thus, the disruption of telomeric chromatin in *Δrif1* cells might result in greater telomere elongation than in *Δrif2* strains by both disrupting repressive Rap1p-Rif1p chromatin and by eliminating the Rif1p-Rif2p interaction that helps recruit more Rif2p to telomeres to inhibit telomerase. Thus, the disruption of both *RIF1* and *RIF2* would abolish both of their functions, resulting in the observed severe, synergistic loss of length control (Wotton and Shore 1997)(Figure 1, lane 15).

Interestingly, the elongated, deregulated telomeres of *tlc1-476A/TLC1* cells showed increased Rif1p and Rif2p association with telomeric DNA in ChIP analyses even though Rap1p association decreased. Rif2p association with telomeric DNA in increased in *Δrif1* cells. We propose that this relative increase in Rif protein telomeric association may be a cellular response that attempts to re-regulate telomeres. Recent high-throughput 2-hybrid studies of Rif2p suggest that it may have as many as 80 binding partners, including Sir2p (Ito, Chiba et al. 2001), raising the possibility that

other protein-protein interactions may also be important for Rif protein recruitment to telomeric chromatin.

This work, taken together with previous studies, provides evidence that telomere chromatin dynamically changes through the cell cycle. While the fundamental signals for telomeric chromatin remodeling have yet to be elucidated, it is likely that cell cycle regulation of other telomeric and general chromatin factors such as the Ku's, SIRs, nucleosomes, and condensins also occurs. It will be of great interest to integrate the behavior of these as well as the damage and replication machineries into a more detailed model of how telomeric chromatin is modulated through the cell cycle to maximally protect the chromosome ends through cell growth, DNA replication, and nuclear division.

Acknowledgements

We would like to thank David Shore for providing the W303-1a and RIF1-9xMYC strains and Judith Berman for providing polyclonal α-Rap1p antibody. We would also like to than Jason Leib for the generous gift of α-Rif2 antibody, permission to reprint his RAP1 and SIR protein chromatin association data in Figure 5, and help advice for analysis of our microarray data. We are grateful to Daniel Levy for sharing unpublished RIF2 results, Inna Botchkina for performing FACS analyses, and David Smith for computer support and assistance with microarray data processing. We also thank Jue Lin for her helpful comments on the manuscript. This work was supported by NIH grant GM26259 and the NSF graduate student fellowship program for Christopher Smith.

References

Biggins, S., N. Bhalla, et al. (2001). "Genes Involved in Sister Chromatid Separation and Segregation in the Budding Yeast Saccharomyces cerevisiae." Genetics **159**(2): 453-70.

Bourns, B. D., M. K. Alexander, et al. (1998). "Sir proteins, Rif proteins, and Cdc13p bind Saccharomyces telomeres in vivo." Mol Cell Biol **18**(9): 5600-8.

Brachmann, C. B., A. Davies, et al. (1998). "Designer deletion strains derived from Saccharomyces cerevisiae S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications." Yeast **14**(2): 115-32.

Buck, S. W. and D. Shore (1995). "Action of a RAP1 carboxy-terminal silencing domain reveals an underlying competition between HMR and telomeres in yeast." Genes Dev **9**(3): 370-84.

Chan, C. S. and B. K. Tye (1983). "Organization of DNA sequences and replication origins at yeast telomeres." Cell **33**(2): 563-73.

Chan, S. W., J. Chang, et al. (2001). "Altering telomere structure allows telomerase to act in yeast lacking ATM kinases." Curr Biol **11**(16): 1240-50.

Cockell, M., F. Palladino, et al. (1995). "The carboxy termini of Sir4 and Rap1 affect Sir3 localization: evidence for a multicomponent complex required for yeast telomeric silencing." J Cell Biol **129**(4): 909-24.

Cohn, M. and E. H. Blackburn (1995). "Telomerase in yeast." Science **269**(5222): 396-400.

Craven, R. J. and T. D. Petes (1999). "Dependence of the regulation of telomere length on the type of subtelomeric repeat in the yeast Saccharomyces cerevisiae." Genetics **152**(4): 1531-41.

Diede, S. J. and D. E. Gottschling (1999). "Telomerase-mediated telomere addition in vivo requires DNA primase and DNA polymerases alpha and delta." Cell **99**(7): 723-33.

Diede, S. J. and D. E. Gottschling (2001). "Exonuclease activity is required for sequence addition and Cdc13p loading at a de novo telomere." Curr Biol **11**(17): 1336-40.

Enomoto, S., P. D. McCune-Zierath, et al. (1997). "RLF2, a subunit of yeast chromatin assembly factor-I, is required for telomeric chromatin function in vivo." Genes Dev **11**(3): 358-70.

Evans, S. K. and V. Lundblad (1999). "Est1 and Cdc13 as comediators of telomerase access." Science **286**(5437): 117-20.

Fink, C. G. a. G. R. (1991). Guide to Yeast Genetics and Molecular Biology. San Diego, Academic Press.

Galy, V., J. C. Olivo-Marin, et al. (2000). "Nuclear pore complexes in the organization of silent telomeric chromatin." Nature **403**(6765): 108-12.

Gerton, J. L., J. DeRisi, et al. (2000). "Inaugural article: global mapping of meiotic recombination hotspots and coldspots in the yeast Saccharomyces cerevisiae." Proc Natl Acad Sci U S A **97**(21): 11383-90.

Gotta, M., T. Laroche, et al. (1996). "The clustering of telomeres and colocalization with Rap1, Sir3, and Sir4 proteins in wild-type Saccharomyces cerevisiae." J Cell Biol **134**(6): 1349-63.

Grandin, N., C. Damon, et al. (2001). "Ten1 functions in telomere end protection and length regulation in association with Stn1 and Cdc13." Embo J **20**(5): 1173-83.

Grunstein, M. (1997). "Molecular model for telomeric heterochromatin in yeast." Curr Opin Cell Biol **9**(3): 383-7.

Guacci, V., E. Hogan, et al. (1994). "Chromosome condensation and sister chromatid pairing in budding yeast." J Cell Biol **125**(3): 517-30.

Hande, M. P., E. Samper, et al. (1999). "Telomere length dynamics and chromosomal instability in cells derived from telomerase null mice." J Cell Biol **144**(4): 589-601.

Hardy, C. F., L. Sussel, et al. (1992). "A RAP1-interacting protein involved in transcriptional silencing and telomere length regulation." Genes Dev **6**(5): 801-14.

Hecht, A., S. Strahl-Bolsinger, et al. (1996). "Spreading of transcriptional repressor SIR3 from telomeric heterochromatin." Nature **383**(6595): 92-6.

Hughes, T. R., S. K. Evans, et al. (2000). "The Est3 protein is a subunit of yeast telomerase." Curr Biol **10**(13): 809-12.

Ito, T., T. Chiba, et al. (2001). "A comprehensive two-hybrid analysis to explore the yeast protein interactome." Proc Natl Acad Sci U S A **98**(8): 4569-74.

Iyer, V. R., C. E. Horak, et al. (2001). "Genomic binding sites of the yeast cell-cycle transcription factors SBF and MBF." Nature **409**(6819): 533-8.

Jager, D. and P. Philippsen (1989). "Stabilization of dicentric chromosomes in Saccharomyces cerevisiae by telomere addition to broken ends or by centromere deletion." Embo J **8**(1): 247-54.

Johnson, E. S., I. Schwienhorst, et al. (1997). "The ubiquitin-like protein Smt3p is activated for conjugation to other proteins by an Aos1p/Uba2p heterodimer." Embo J **16**(18): 5509-19.

Kennedy, B. K., M. Gotta, et al. (1997). "Redistribution of silencing proteins from telomeres to the nucleolus is associated with extension of life span in S. cerevisiae." Cell **89**(3): 381-91.

Klein, F., T. Laroche, et al. (1992). "Localization of RAP1 and topoisomerase II in nuclei and meiotic chromosomes of yeast." J Cell Biol **117**(5): 935-48.

Laroche, T., S. G. Martin, et al. (2000). "The dynamics of yeast telomeres and silencing proteins through the cell cycle." J Struct Biol **129**(2-3): 159-74.

Lendvay, T. S., D. K. Morris, et al. (1996). "Senescence mutants of Saccharomyces cerevisiae with a defect in telomere replication identify three additional EST genes." Genetics **144**(4): 1399-412.

Lieb, J. D., X. Liu, et al. (2001). "Promoter-specific binding of Rap1 revealed by genome-wide maps of protein-DNA association." Nat Genet **28**(4): 327-34.

Loidl, J., K. Nairz, et al. (1991). "Meiotic chromosome synapsis in a haploid yeast." Chromosoma **100**(4): 221-8.

Longtine, M. S., A. McKenzie, 3rd, et al. (1998). "Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae." Yeast **14**(10): 953-61.

Louis, E. J. and J. E. Haber (1990). "The subtelomeric Y' repeat family in Saccharomyces cerevisiae: an experimental system for repeated sequence evolution." Genetics **124**(3): 533-45.

Louis, E. J. and J. E. Haber (1992). "The structure and evolution of subtelomeric Y' repeats in Saccharomyces cerevisiae." Genetics **131**(3): 559-74.

Lundblad, V. and J. W. Szostak (1989). "A mutant with a defect in telomere elongation leads to senescence in yeast." Cell **57**(4): 633-43.

Marcand, S., V. Brevet, et al. (2000). "Cell cycle restriction of telomere elongation." Curr Biol **10**(8): 487-90.

Marcand, S., S. W. Buck, et al. (1996). "Silencing of genes at nontelomeric sites in yeast is controlled by sequestration of silencing factors at telomeres by Rap 1 protein." Genes Dev **10**(11): 1297-309.

Martin, S. G., T. Laroche, et al. (1999). "Relocalization of telomeric Ku and SIR proteins in response to DNA strand breaks in yeast." Cell **97**(5): 621-33.

McClintock, B. (1941). "The stability of broken ends of chromosomes in *Zea Mays*." Genetics **26**: 234-82.

Michaelis, C., R. Ciosk, et al. (1997). "Cohesins: chromosomal proteins that prevent premature separation of sister chromatids." Cell **91**(1): 35-45.

Mishra, K. and D. Shore (1999). "Yeast Ku protein plays a direct role in telomeric silencing and counteracts inhibition by rif proteins." Curr Biol **9**(19): 1123-6.

Moazed, D. and D. Johnson (1996). "A deubiquitinating enzyme interacts with SIR4 and regulates silencing in S. cerevisiae." Cell **86**(4): 667-77.

Moretti, P., K. Freeman, et al. (1994). "Evidence that a complex of SIR proteins interacts with the silencer and telomere-binding protein RAP1." Genes Dev **8**(19): 2257-69.

Moretti, P. and D. Shore (2001). "Multiple interactions in sir protein recruitment by rap1p at silencers and telomeres in yeast." Mol Cell Biol **21**(23): 8082-94.

Muller, H. J. (1938). "The remaking of chromosomes." Collecting Net **8**: 182-95,198.

Nilsson, B., T. Moks, et al. (1987). "A synthetic IgG-binding domain based on staphylococcal protein A." Protein Eng **1**(2): 107-13.

Nugent, C. I., G. Bosco, et al. (1998). "Telomere maintenance is dependent on activities required for end repair of double-strand breaks." Curr Biol **8**(11): 657-60.

Pennock, E., K. Buckley, et al. (2001). "Cdc13 delivers separate complexes to the telomere for end protection and replication." Cell **104**(3): 387-96.

Peterson, S. E., A. E. Stellwagen, et al. (2001). "The function of a stem-loop in telomerase RNA is linked to the DNA repair protein Ku." Nat Genet **27**(1): 64-7.

Qi, H. and V. A. Zakian (2000). "The Saccharomyces telomere-binding protein Cdc13p interacts with both the catalytic subunit of DNA polymerase alpha and the telomerase- associated est1 protein." Genes Dev **14**(14): 1777-88.

Raghuraman, M. K., E. A. Winzeler, et al. (2001). "Replication dynamics of the yeast genome." Science **294**(5540): 115-21.

Ritchie, K. B. and T. D. Petes (2000). "The Mre11p/Rad50p/Xrs2p complex and the Tel1p function in a single pathway for telomere maintenance in yeast." Genetics **155**(1): 475-9.

Sandell, L. L. and V. A. Zakian (1993). "Loss of a yeast telomere: arrest, recovery, and chromosome loss." Cell **75**(4): 729-39.

Schwarz, S. E., K. Matuschewski, et al. (1998). "The ubiquitin-like proteins SMT3 and SUMO-1 are conjugated by the UBC9 E2 enzyme." Proc Natl Acad Sci U S A **95**(2): 560-4.

Seto, A. G., A. J. Zaug, et al. (1999). "Saccharomyces cerevisiae telomerase is an Sm small nuclear ribonucleoprotein particle." Nature **401**(6749): 177-80.

Seufert, W., B. Futcher, et al. (1995). "Role of a ubiquitin-conjugating enzyme in degradation of S- and M-phase cyclins." Nature **373**(6509): 78-81.

Shore, D. (1994). "RAP1: a protean regulator in yeast." Trends Genet **10**(11): 408-12.

Smith, C. D. and E. H. Blackburn (1999). "Uncapping and deregulation of telomeres lead to detrimental cellular consequences in yeast." J Cell Biol **145**(2): 203-14.

Smith, J. S., C. B. Brachmann, et al. (1998). "Distribution of a limited Sir2 protein pool regulates the strength of yeast rDNA silencing and is modulated by Sir4p." Genetics **149**(3): 1205-19.

Spellman, P. T., G. Sherlock, et al. (1998). "Comprehensive identification of cell cycle-regulated genes of the yeast Saccharomyces cerevisiae by microarray hybridization." Mol Biol Cell **9**(12): 3273-97.

Strahl-Bolsinger, S., A. Hecht, et al. (1997). "SIR2 and SIR4 interactions differ in core and extended telomeric heterochromatin in yeast." Genes Dev **11**(1): 83-93.

Teng, S. C., J. Chang, et al. (2000). "Telomerase-independent lengthening of yeast telomeres occurs by an abrupt Rad50p-dependent, Rif-inhibited recombinational process." Mol Cell **6**(4): 947-52.

Tsukamoto, Y., A. K. Taggart, et al. (2001). "The role of the Mre11-Rad50-Xrs2 complex in telomerase- mediated lengthening of Saccharomyces cerevisiae telomeres." Curr Biol **11**(17): 1328-35.

Uetz, P. and R. E. Hughes (2000). "Systematic and large-scale two-hybrid screens." Curr Opin Microbiol **3**(3): 303-8.

Wellinger, R. J., K. Ethier, et al. (1996). "Evidence for a new step in telomere maintenance." Cell **85**(3): 423-33.

Wellinger, R. J., A. J. Wolf, et al. (1993). "Saccharomyces telomeres acquire single-strand TG1-3 tails late in S phase." Cell **72**(1): 51-60.

Wotton, D. and D. Shore (1997). "A novel Rap1p-interacting factor, Rif2p, cooperates with Rif1p to regulate telomere length in Saccharomyces cerevisiae." Genes Dev **11**(6): 748-60.

Wright, J. H., D. E. Gottschling, et al. (1992). "Saccharomyces telomeres assume a non-nucleosomal chromatin structure." Genes Dev **6**(2): 197-210.

Wu, G., W. H. Lee, et al. (2000). "NBS1 and TRF1 colocalize at promyelocytic leukemia bodies during late S/G2 phases in immortalized telomerase-negative cells. Implication of NBS1 in alternative lengthening of telomeres." J Biol Chem **275**(39): 30618-22.

Yamada, M., N. Hayatsu, et al. (1998). "Y'-Help1, a DNA helicase encoded by the yeast subtelomeric Y' element, is induced in survivors defective for telomerase." J Biol Chem **273**(50): 33360-6.

Zhu, X. D., B. Kuster, et al. (2000). "Cell-cycle-regulated association of RAD50/MRE11/NBS1 with TRF2 and human telomeres." Nat Genet **25**(3): 347-52.

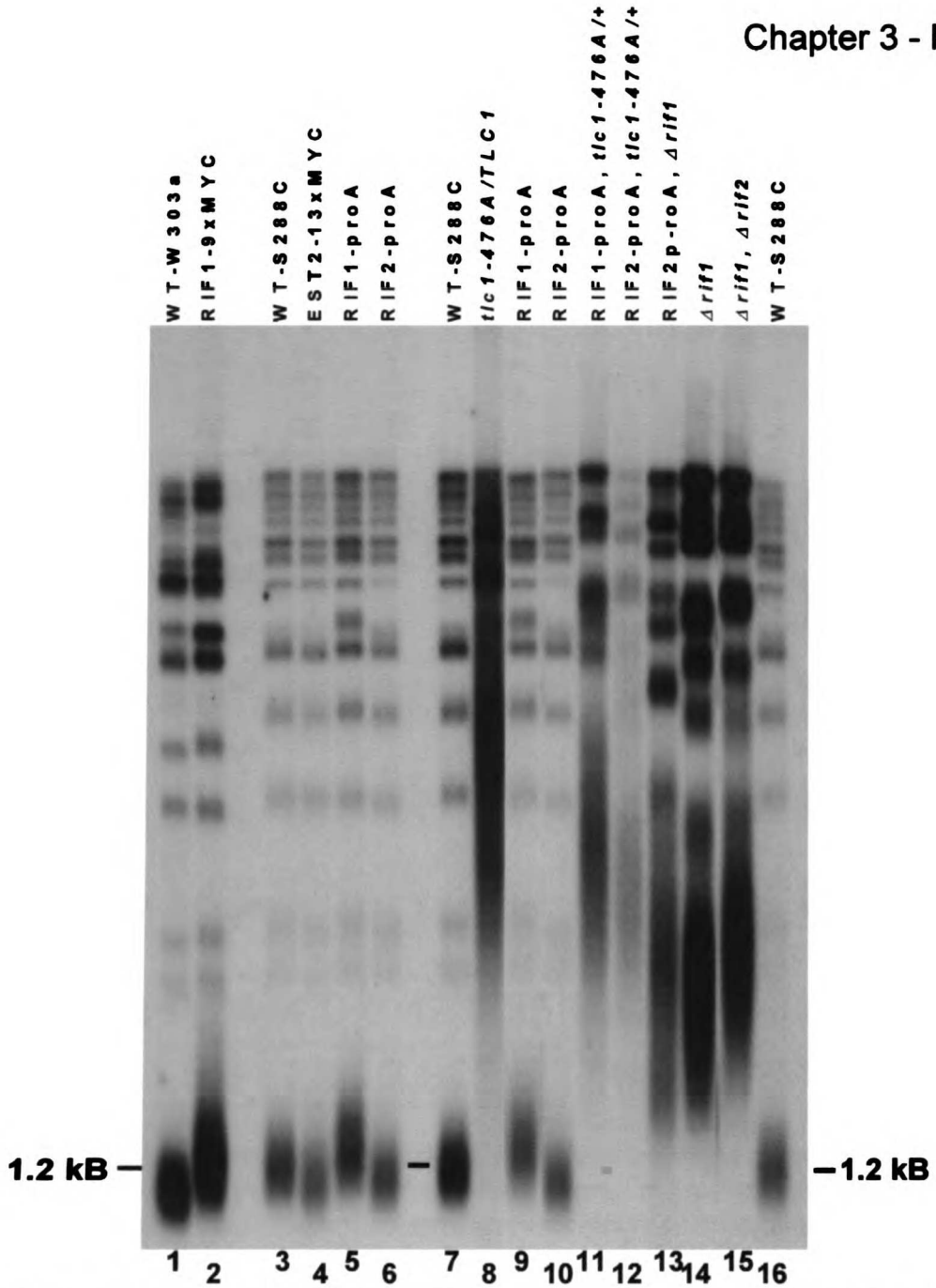


Figure 1- Telomere length phenotypes of epitope-tagged and *tlc1-476A/TLC1* heteroallelic strains. Genomic DNAs were purified from haploid strains and heteroallelic *tlc1-476A/TLC1* strains. Epitope-tagged strains do not show significant telomere lengthening or shortening (lanes 1-6). Strains containing *tlc1-476A/TLC1* show elongated, deregulated telomeres. WT strains of both the W303a and S288C strain backgrounds are shown (lanes 1, 3, 7, 16). *RIF1* and *RIF1, RIF2* deletion strains are also shown for reference (lanes 14, 15). The *Δrif1*, *Δrif2* strain used in this study (lane 15) was not extensively passaged and did not have fully elongated telomeres. The majority of Y' telomeres migrate with the 1.2 kB shown.

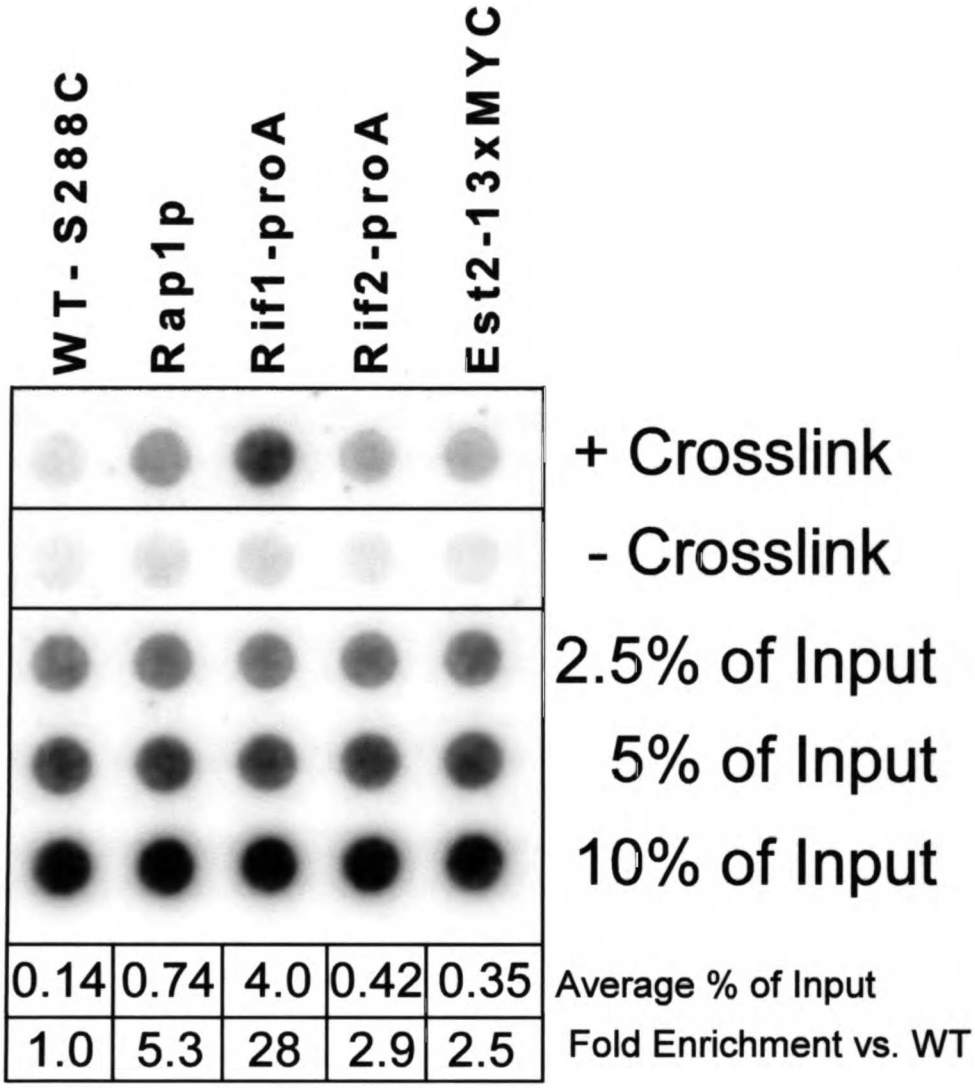
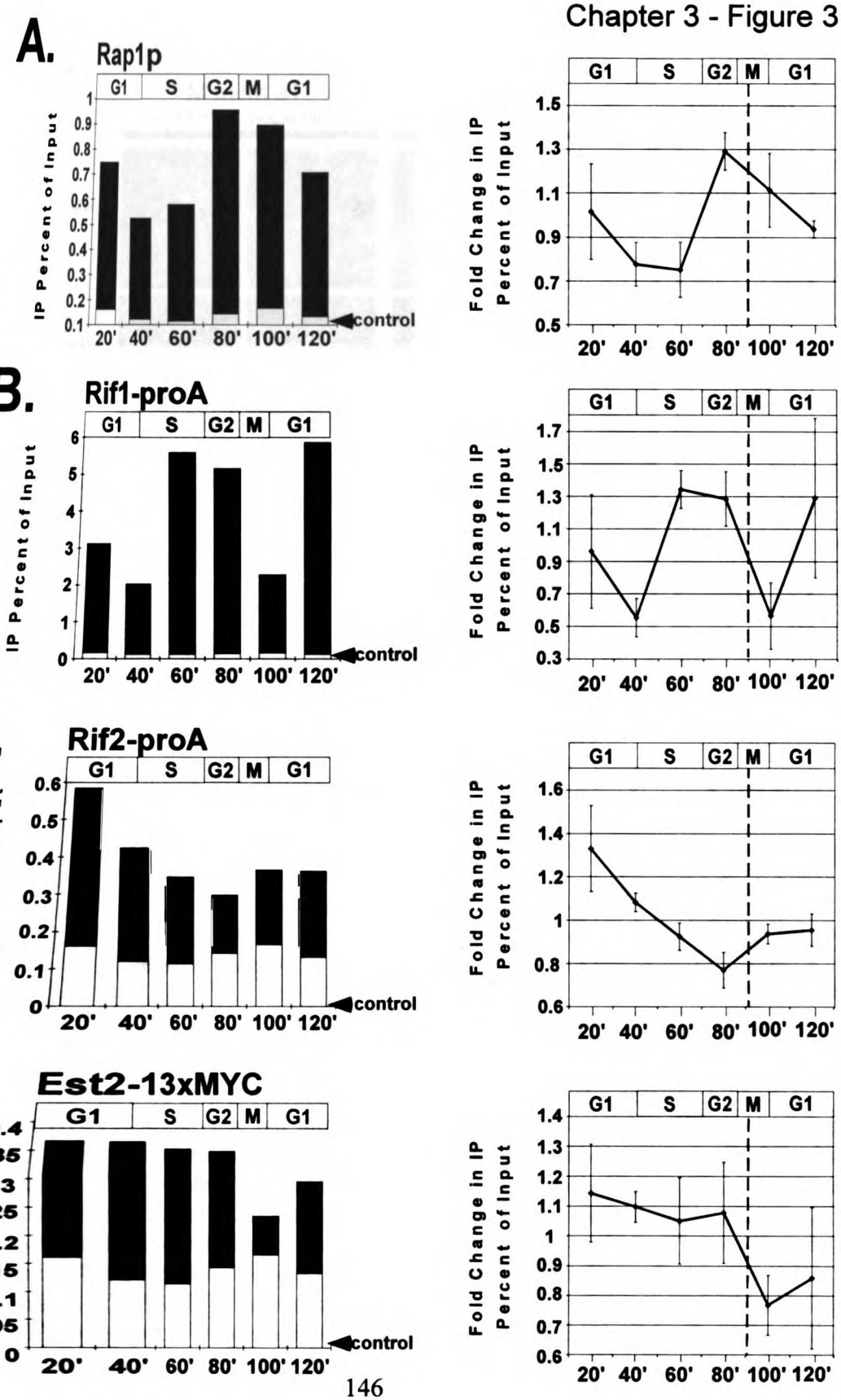


Figure 2 - Chromatin immunoprecipitation of telomeric proteins is dependent upon the presence of crosslinking agent. A representative dotblot of ChIP samples is shown. Asynchronous yeast cultures were chromatin immunoprecipitated both in the presence (row 1) and absence (row 2) of 1% formaldehyde for 15 minutes at room temperature. The average percent of total telomeric DNA IP'd is shown at the bottom as well as the fold-enrichment over control strains that were either mock-IP'd without primary antibody, or contained no epitope tag. The numbers shown for the averages represent the amount of telomeric DNA IP'd over the entire timecourse for all replicates and do not reflect the representative blot shown above.

Figure 3 (next page) - Telomeric proteins change their crosslinkability to telomeric DNA during the cell cycle. α-factor synchronized cultures were released into the cell cycle and 1% formaldehyde fixed at 20 minute intervals. Rap1p (A), Rif1-proA (B) Rif2-proA (C), and Est2-13xMYC (D) samples were ChIP'd and associated DNA was probed on dotblots with a telomeric oligo. Raw telomeric signal as a percentage of total input DNA is shown in left-hand panel (black bars), with control "mock" IPs or untagged controls are superimposed (white bars, indicated by black arrowheads). The approximate stage of the cell cycle, as determined by budding indices is shown above graphs. Right-hand panels show fold change in telomeric signals that have been normalized to the average signal for all timepoints in the timecourse. Standard deviation of the means are shown. Dashed line represents the midpoint of mitosis.

Chapter 3 - Figure 3



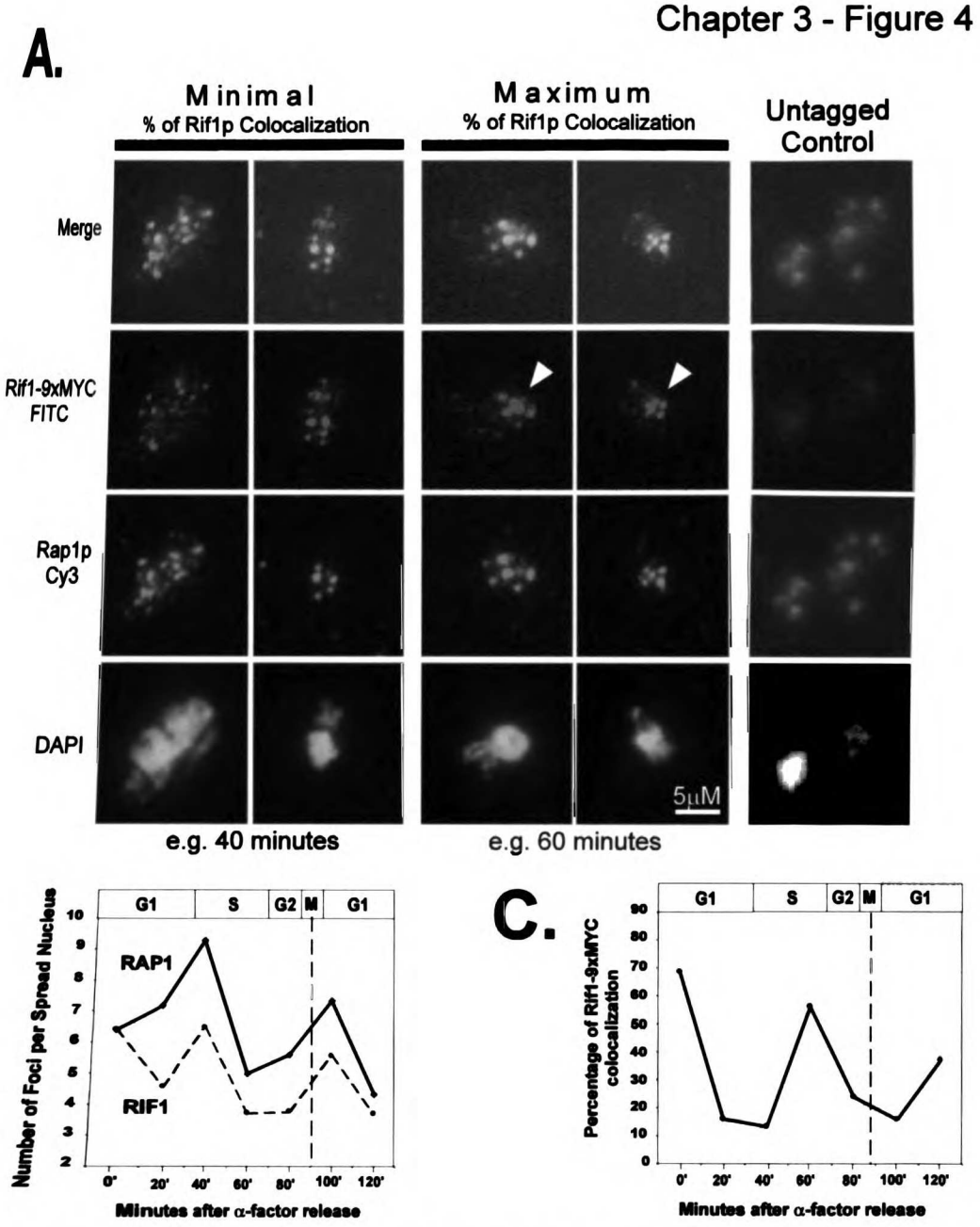
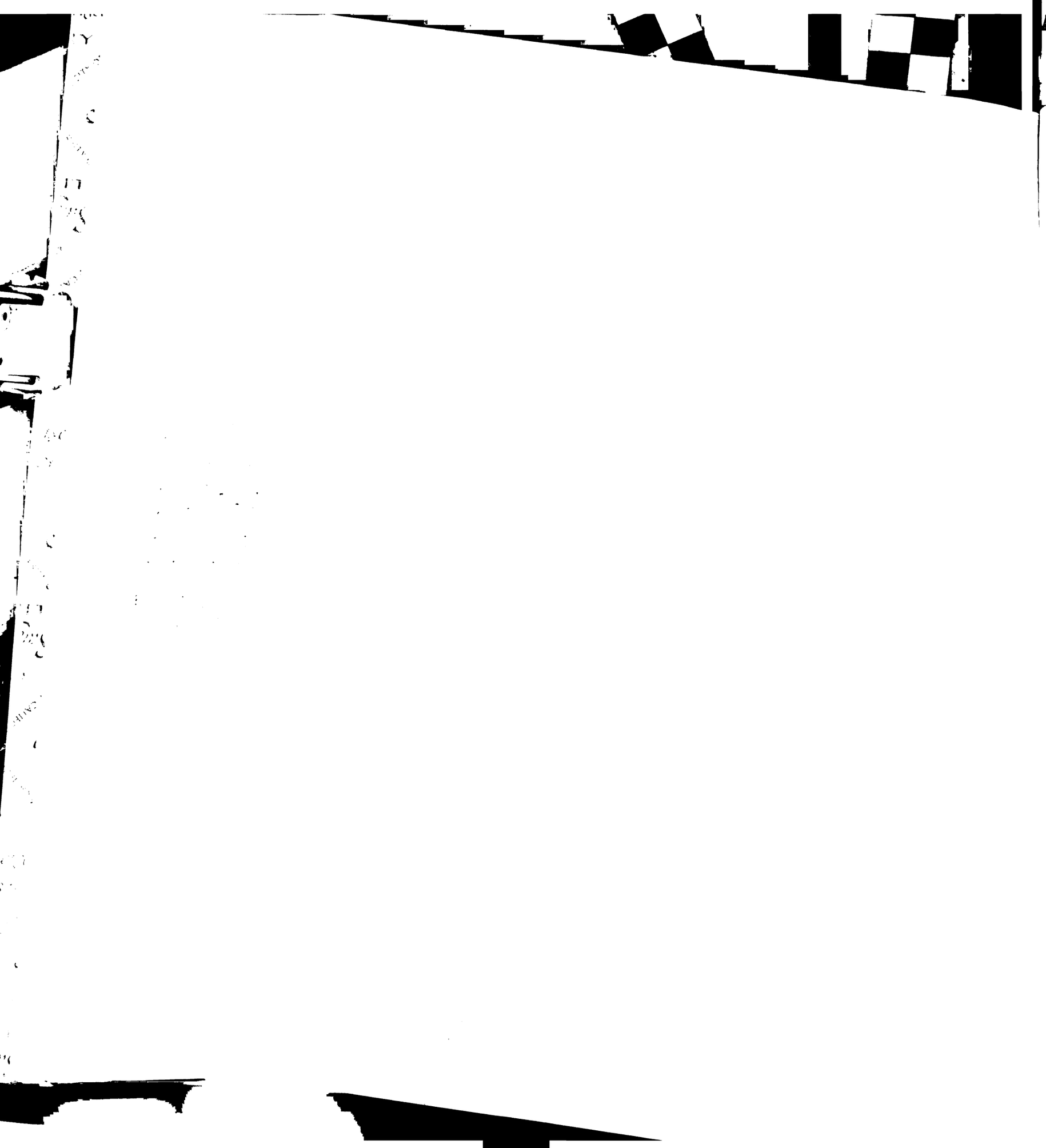


Figure 4 - Rap1p and Rif1p colocalize and cluster through the cell cycle. α-factor synchronized cultures were released into the cell cycle and collected at 20 minute intervals. Spheroplasts were spread on glass slides and immunofluorescence performed for Rap1p (Cy3, red) and Rif1-9xMYC (FITC, green). Representative examples of maximally colocalized and minimally colocalized spots are shown as well as untagged control (A). White arrowheads indicate large, clustered Rif1-9xMYC foci (A). The average number of Rap1p and Rif1-9xMYC foci per nucleus was quantified for each timepoint (B). The percentage of Rif1-9xMYC colocalization to Rap1p through the cell cycle was also quantified (C). The approximate stage of the cell cycle, as determined by budding indices is shown above graphs. Dashed line in B and C represents the midpoint of mitosis.



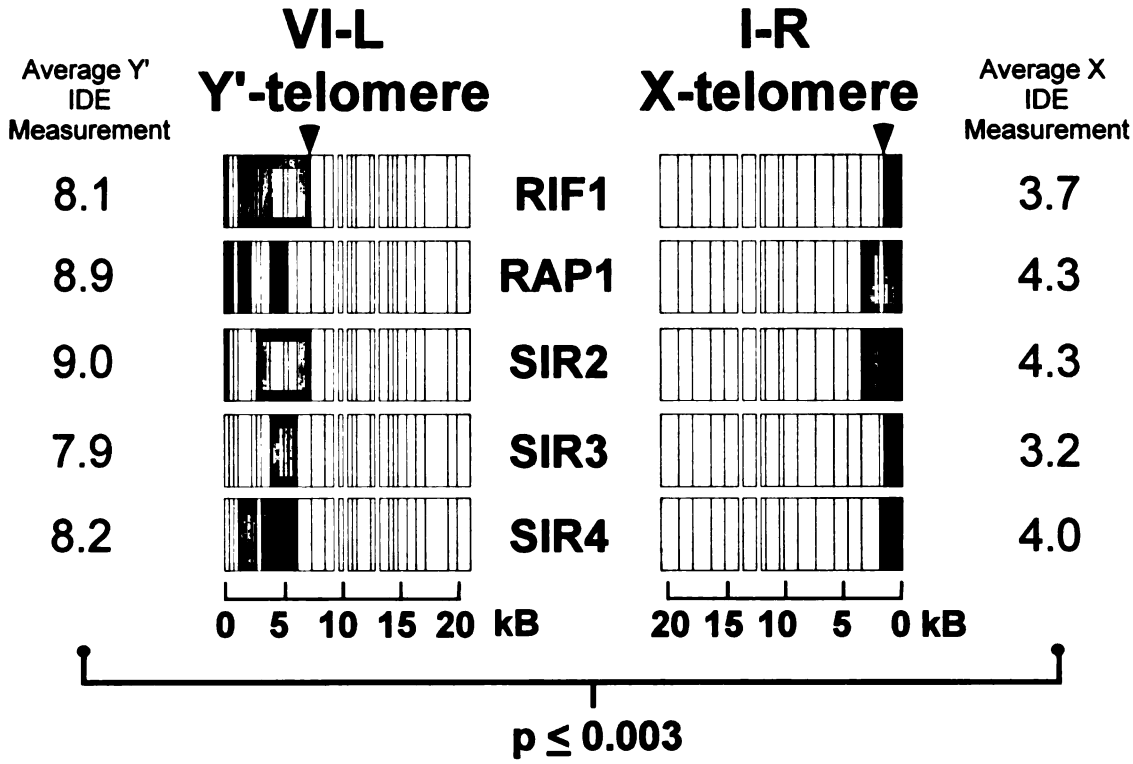


Figure 5 - RIF1, RAP1, and SIRs exhibit a broader region of association to Y' telomeres than X telomeres. Rif1-proA Innermost Distance from the End (IDE) measurements. Multiple intergenic microarrays were hybridized with Rif1-proA ChIP samples from asynchronous cultures. The IDE was measured for targets in the top 5% of IP'd Rif1-proA fragments. RAP1, SIR2-4 data is reproduced from (Lieb, Liu et al. 2001) and shown for comparison purposes. Physical maps of representative chromosome ends are show for X-element (I-R) and Y' element (VI-L) telomeres for RIF1, RAP1, and the SIRs. Dark gray indicates association detected on the microarray, while light gray indicates regions where no association was detected. Scale bars are shown at bottom. The Rif1-proA, Rap1p, and Sir2-4 protein average IDE measurements in kB are shown for chromosomes with X-elements or Y' elements. A representative innermost associated DNA fragment is indicated by black arrowhead. A statistically significant length difference for Rif1-proA IDE measurements of X- and Y' element chromosomes is indicated. Rap1p and the Sir2-4 proteins were similarly significant.

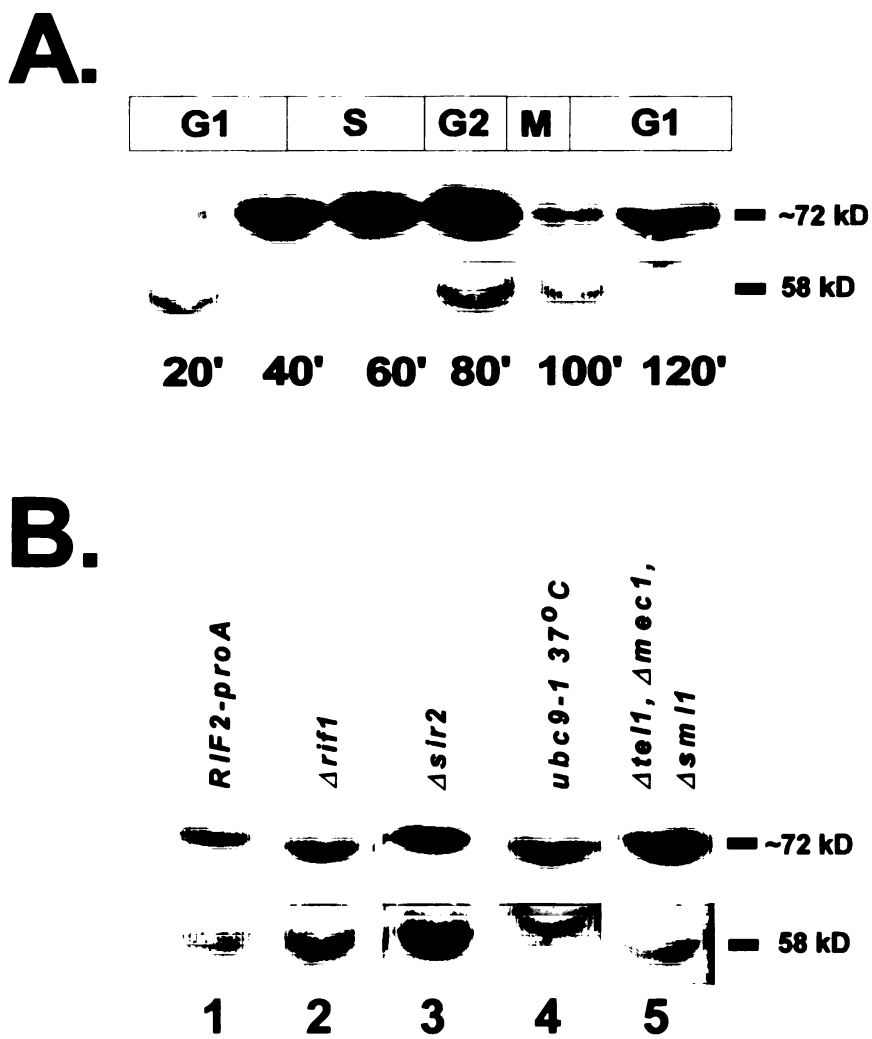


Figure 6 - Rif2p exists as a doublet that is modified through the cell cycle. α-factor synchronized cultures were released into the cell cycle and collected at 20 minute intervals. Rif2-proA lysates were IP'd with IgG sepharose, resolved on SDS-PAGE gels, and detected with α-Rif2 antibody (A). The approximate sizes of Rif2-proA species are indicated. The approximate stage of the cell cycle, as determined by budding indices is shown at the top (A). Genetic backgrounds checked for modulation of the Rif2-proA doublet in asynchronous cultures are shown in B. Optimal linear exposures from the same gel were used to highlight changes in the ratio of the doublet.

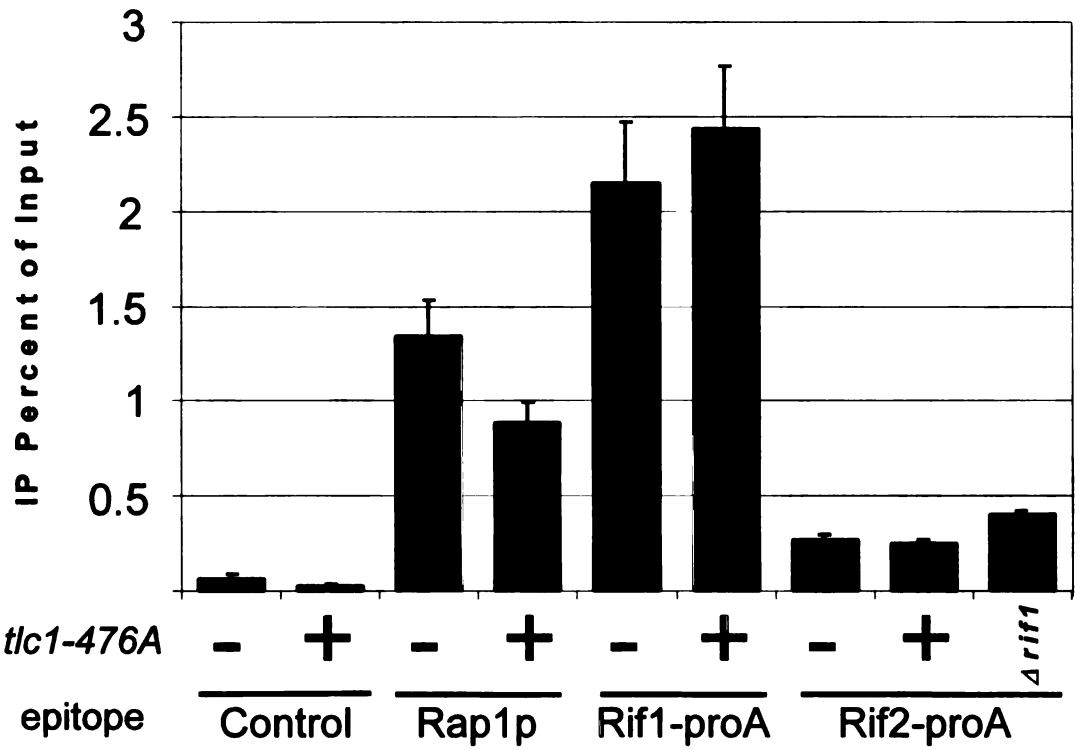


Figure 7 - Rif proteins increase their association to deregulated telomeres, while Rap1p does not ChIP assays were performed on asynchronous haploid cultures that had either *TLC1* (-) or *tlc1-476A/TLC1* (+) at the *TLC1* locus, or contained a deletion at the *RIF1* locus. Epitopes detected are indicated. Antibodies used for each strain are described in Materials & Methods. The percentage of IP'd telomeric DNA as a function of total telomeric DNA input is shown.

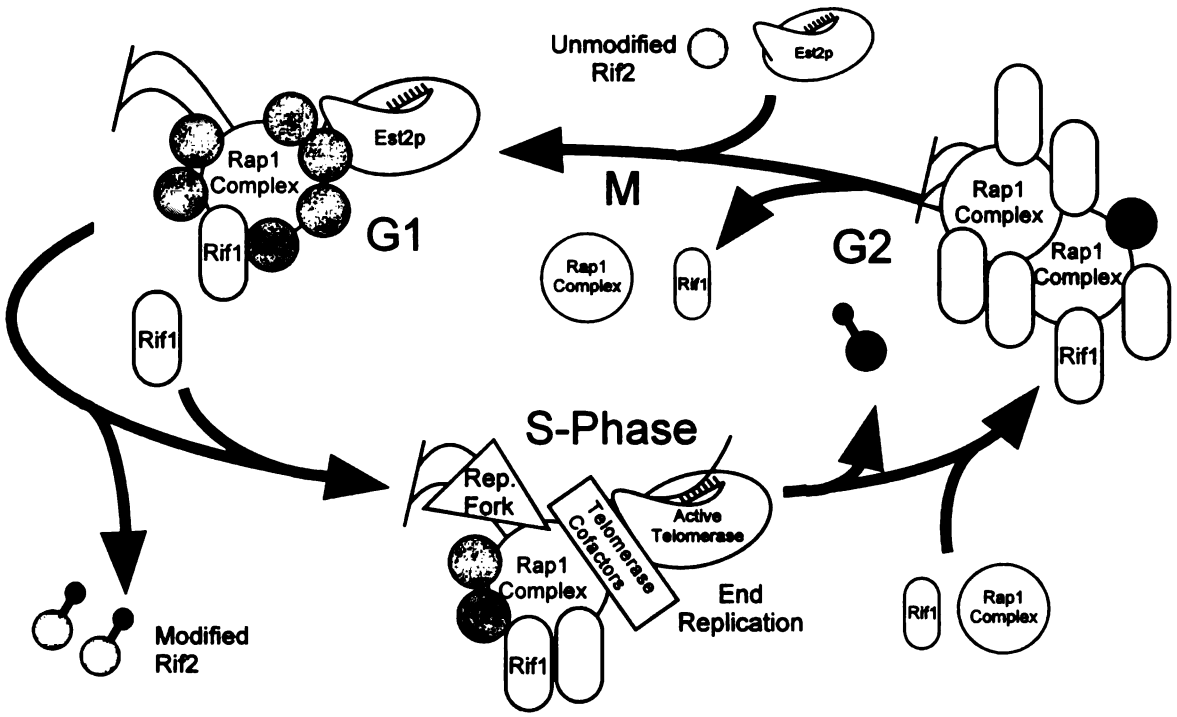


Figure 8 - A model for telomeric chromatin remodeling through the cell cycle. A speculative model showing the dynamics of telomeric remodeling during the cell cycle. Briefly, increasing modification of Rif2p (small shaded circles) through S-phase results in its displacement from telomeric DNA, thus releasing inhibition of telomerase and its associated co-factors (rectangle). Telomerase, its co-factors, and replication forks (triangle) elongate telomeres in S-phase and are displaced through G2 when Rap1p (large circles) and Rif1p (rounded blocks) are maximally associated. As cells undergo mitosis, Rif1p strongly disassociates from telomeres, allowing unmodified Rif2p to rebind chromatin through M or the subsequent G1 phase.

SUPPLEMENTAL DATA

The Supplemental Data for Chapter 3 are contained in Excel and text format in the CD supplement that comprises Appendix 4. Please see Appendix 4 for a complete file list of the CD supplement.

Figure List for Supplemental Data for Chapter 3

A – Strain Table

B - Normalization of Rif2 and Est2 ChIP timecourse data to WT signal

C - IDE Calculations for unique, redundant, and complete data sets

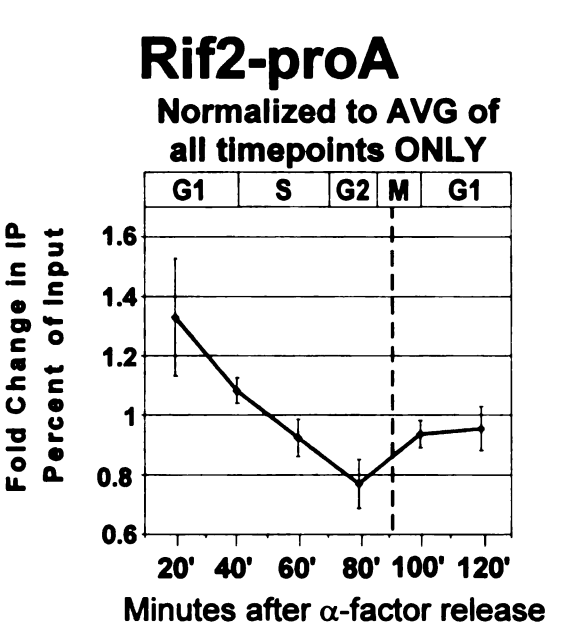
D - Top 10% ranking targets IP'd by Rif1

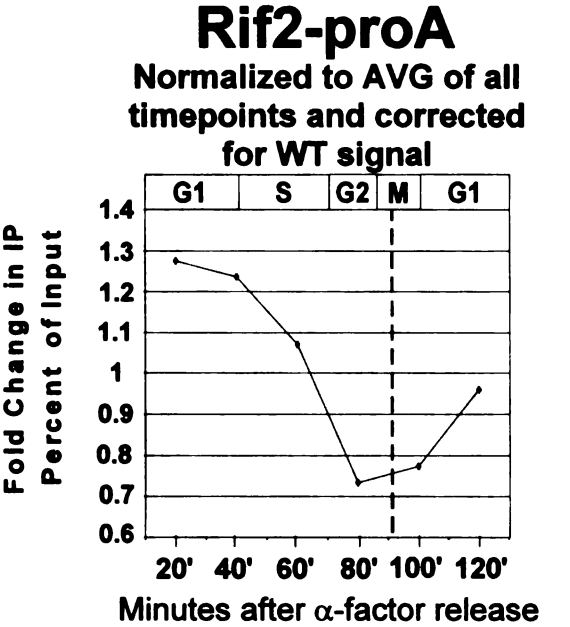
E - Common targets IP'd by both Rif1 and Rap1

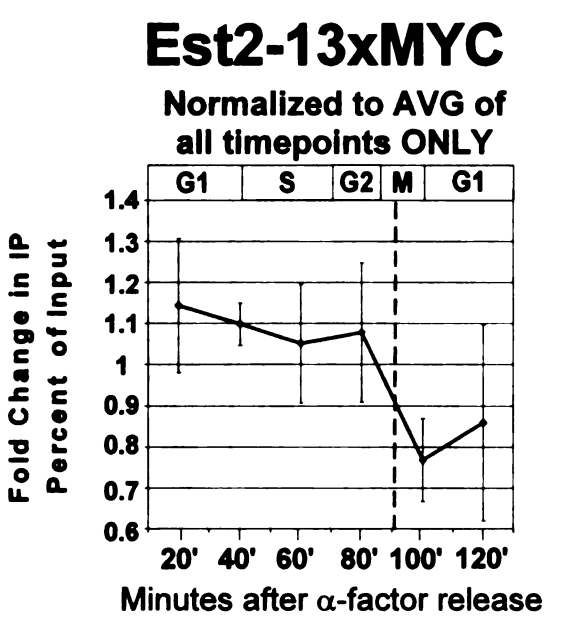
F - Targets IP'd by Rif1 but not Rap1

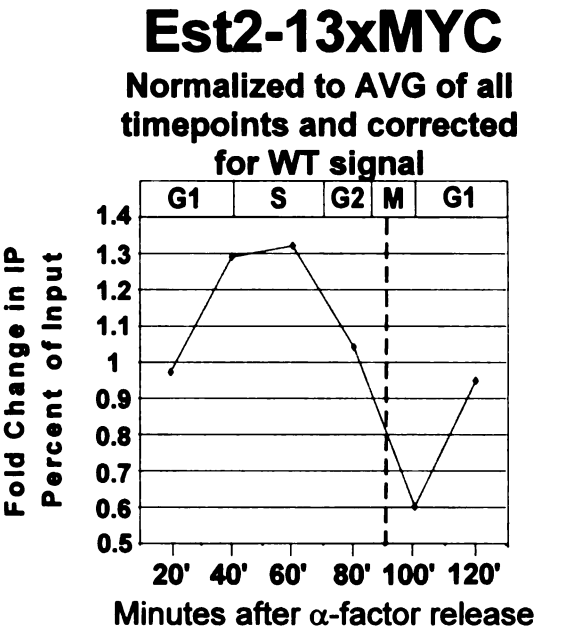
Strains

Description	Parent	Genotype												
WT S288C	BY4736	MAT a	ade2Δ::hisG	his3Δ200	met15Δ0	trp1Δ63	ura3Δ0							
WT S288C, bar1	BY4736	MAT a	bar1::URA3	ade2Δ::hisG	his3Δ200	met15Δ0	trp1Δ63	ura3Δ0						
tlc1-476A-1/TLC1	BY4736	MAT a	bar1::URA3	tlc1-476A::HIS3::TLC1	ade2Δ::hisG	his3Δ200	met15Δ0	trp1Δ63	ura3Δ0					
RIF1-ProA	BY4736	MAT a	bar1	RIF1-2xProA::HISMX6	ade2Δ::hisG	his3Δ200	met15Δ0	trp1Δ63	ura3Δ0					
RIF1-ProA, 476A-1/TLC1	BY4736	MAT a	bar1	RIF1-2xProA::HISMX6	tlc1-476A::HIS3::TLC1	ade2Δ::hisG	his3Δ200	met15Δ0	trp1Δ63	ura3Δ0				
RIF2-ProA	BY4736	MAT a	bar1	RIF2-2xProA::HISMX6	ade2Δ::hisG	his3Δ200	met15Δ0	trp1Δ63	ura3Δ0					
RIF2-proA, tlc1-476A-1/TLC1	BY4736	MAT a	bar1	RIF2-2xProA::HISMX6	tlc1-476A::HIS3::TLC1	ade2Δ::hisG	his3Δ200	met15Δ0	trp1Δ63	ura3Δ0				
RIF2-proA Δrif1	BY4736	MAT a	bar1	RIF2-2xProA::HISMX6	rif1::TRP1	ade2Δ::hisG	his3Δ200	met15Δ0	trp1Δ63	ura3Δ0				
EST2-13xMYC	BY4736	MAT a	bar1::URA3	EST2-13xMYC::KANMX	ade2Δ::hisG	his3Δ200	met15Δ0	trp1Δ63	ura3Δ0					
Δrif1	BY4705	MAT α	rif1::TRP1	ade2Δ::hisG	met15Δ0	trp1Δ63	ura3Δ0	leu2Δ0	his3Δ200	lys2Δ0				
Δrif1, Δrif2	BY4705	MAT a	rif1::TRP1, rif2::KANMX4	ade2Δ::hisG	met15Δ0	trp1Δ63	ura3Δ0	leu2Δ0	his3Δ200	lys2Δ0				
RIF2-ProA, Δsir2	BY4736	MAT a	bar1	RIF2-2xProA::HISMX6	Dsir2::LEU2	leu2::TRP1	ade2Δ::hisG	his3Δ200	met15Δ0	trp1Δ63	ura3Δ0			
RIF2-ProA, ubc9-1	BY4736	MAT a	bar1	RIF2-2xProA::HISMX6	ubc9-1::TRP1	Δubc9::LEU2	ade2Δ::hisG	his3Δ200	met15Δ0	trp1Δ63	ura3Δ0			
RIF2-ProA, Δlel1, Δmec1, Δsml1	BY4705	MAT a	bar1	RIF2-2xProA::HISMX6	tel1::HIS3	mec1::LEU2	sml1::TRP1	ade2Δ::hisG	his3Δ200	met15Δ0	trp1Δ63	ura3Δ0		
WT W303-1a	Misra & Shore GD 1997	MAT a	bar1	lys2Δ0	ade2-1	his3-11	can1-100	trp3-1	ura3-1	leu2-1				
RIF1-9xMYC	Misra & Shore GD 1997	MAT a	bar1::LEU2	rif1::RIF1-9xMYC::kiTRP	lys2Δ0	ade2-1	his3-11	can1-100	trp3-1	ura3-1	leu2-1			


Rif2-proA
Normalized to AVG of all timepoints ONLY
G1
S
G2
M
G1
Fold Change in IP
Percent of Input
1.6
1.4
1.2
1
0.8
0.6
20' 40' 60' 80' 100' 120'
Minutes after α-factor release


Rif2-proA
Normalized to AVG of all timepoints and corrected for WT signal
G1
S
G2
M
G1
Fold Change in IP
Percent of Input
1.4
1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
20' 40' 60' 80' 100' 120'
Minutes after α-factor release


Est2-13xMYC
Normalized to AVG of all timepoints ONLY
G1
S
G2
M
G1
Fold Change in IP
Percent of Input
1.4
1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
20' 40' 60' 80' 100' 120'
Minutes after α-factor release


Est2-13xMYC
Normalized to AVG of all timepoints and corrected for WT signal
G1
S
G2
M
G1
Fold Change in IP
Percent of Input
1.4
1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
20' 40' 60' 80' 100' 120'
Minutes after α-factor release

Innermost Distance from End (IDE) Measurements Using All End Data

Left Telomeres Chr. #	RAP1	RIF1	SIR2	SIR3	SIR4
1	1807	3435	3435	1807	2169
2	8848	8177	9583	8482	9518
3	5392	2816	4065	3964	4065
4					
5	8786	7230	7230	7230	7230
6	7565	5521	7565	6426	6426
7	3932	6860			
8	7545	6400	8224	6400	6400
9	9696	9155	9500	8793	9500
10	9138	8776	10306	8776	9138
11	2722	799	2722	1811	1811
12	14648	13008	14648	13083	14648
13	8383	7244	7244	7244	7244
14	8330	12208	8330	8330	8330
15	2078	11548	3986	1647	3986
16	9557	7933	9557	7933	7933
Average for Arm	7228	7407	7600	6566	7028
Total Average of X's	**4250**	**4211**	**4312**	**3207**	**3962**
Total Average of Y's	**8926**	**8113**	**8960**	**7929**	**8247**
Total Average	**6777**	**6369**	**7017**	**6280**	**6552**

Right Telomeres Chr.#	RAP1	RIF1	SIR2	SIR3	SIR4
1	2375	2475	2475	1373	2475
2	2844	4130	4127		
3		9029			
4	9915	8277	11191	8277	8639
5	8210	8210	9194	8210	8210
6	590	469	1046	5903	1046
7	10898	8475	10898	8475	8475
8	8253	6682	9147	8253	8253
9	6500				
10	2903	1752	2903	1752	1752
11	15000		15000	13524	15000
12	6274	4896	6274	4896	6274
13	3030	1416	1987	937	1987
14	2415	2051	2415	2051	2415
15	9702	9752	8573	7998	7998
16	5982	5982	4854	5982	5982
	6326	5257	6435	5972	6039

Innermost Distance from End (IDE) Measurements Using Redundant or Unique Data

Redundant End Data			Unique End Data				
Left Arm	Right Arm	Arm Type	Chr.	Arm Type	Left Arm	Right Arm
	893	X	1	X	3435	2475
8177		YS	2	X	7151	4130
2219	1638	X	3	X	2816	9029
		X	4	YL		8277
13720	8210	YL	5	YL	6464	7145
836	469	YS	6		5521	
1934		X	7	YS	6860	8475
		YS	8	YS	12500	14539
		YL	9	X	9155	
		YL	10	X	8776	1752
		X	11	X	799	
		YS2	12	YL2	13083	4896
		YS	13	X	7244	1416
		YL	14	X	12208	2051
11548	7998	X	15	YL	1338	9702
	5982	YL	16	YS	7933	
Average for Arm	6405	4198		Average for Arm	7019	6157
Total Average of X's	**3646**			**Total Average of X's**	**3282**	
Total Average of Y's	**7487**			**Total Average of Y's**	**8942**	
Total Average	**5302**			**Total Average**	**6636**	

Top 10% Ranking Targets IP'd by Rif1-proA

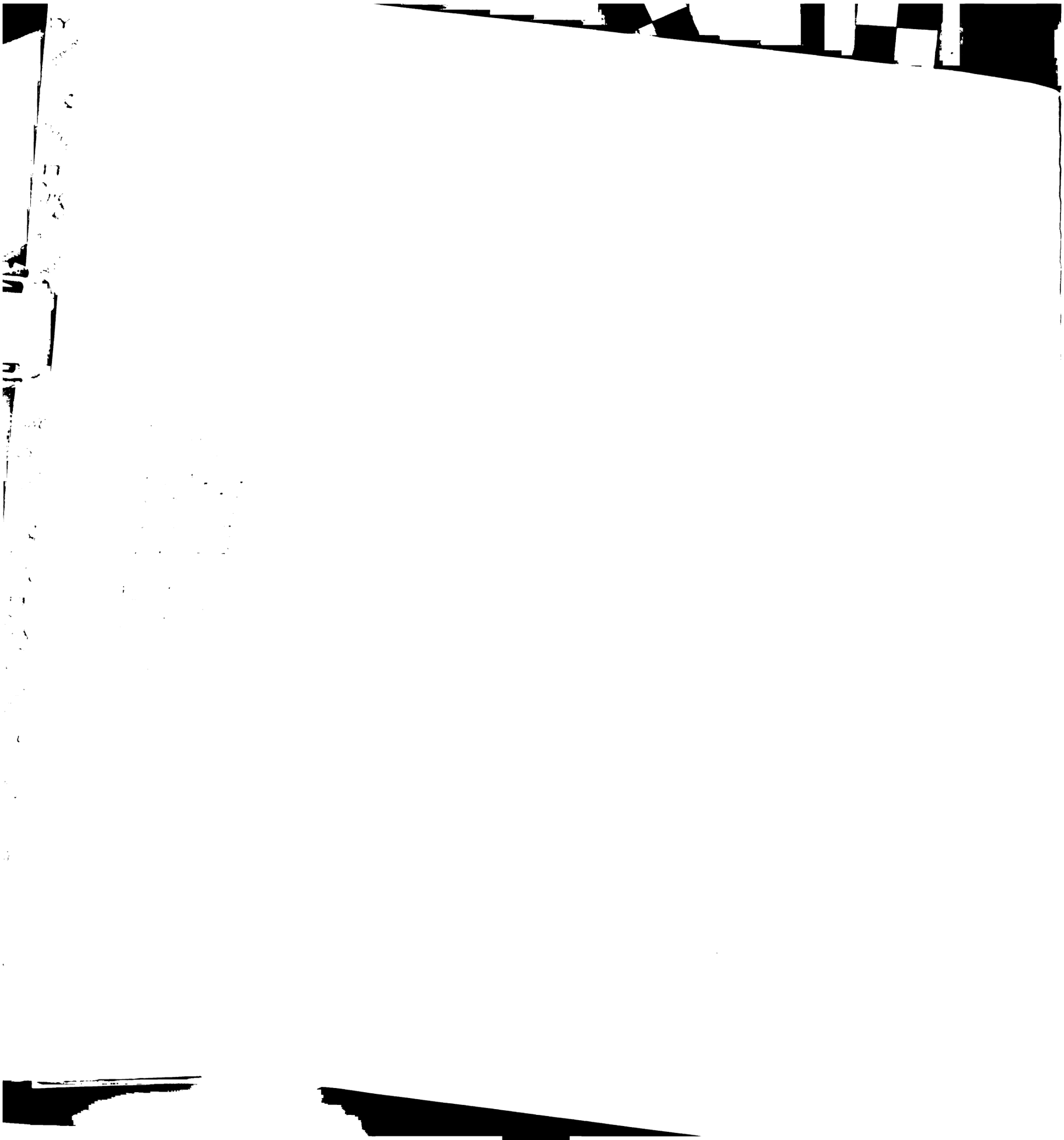
Feature	Rank
iYPL193W	1
YDL176W	1
iYMR137C	1
iYNL338W	0.999
iYHR217C	0.999
YHL027W	0.998
iYJL089W	0.998
YBR279W	0.997
YDR545W	0.997
iYJL225C-1	0.997
YPL058C	0.996
iYHR216W	0.996
YER011W	0.995
YIL173W	0.995
iYEL004W	0.994
YHRCTy1-1D	0.994
iYPR148C-1	0.994
YHR217C	0.994
iYPL212C	0.994
TEL1R	0.994
iYNL339C	0.994
iYERWomega2-0	0.994
YMR326C	0.993
YHL039W	0.993
iYHR219W	0.993
iYLL065W	0.992
iYIR010W	0.992
YDR248C	0.9915
YCL018W	0.991
YPR168W	0.991
YDR170C	0.991
YJR162C	0.991
iYDR542W	0.991
iYHL049C-1	0.991
iYPR035W	0.9905
iYJL225C-0	0.99
YNL337W	0.99
YER166W	0.99
YGR098C	0.989
YDR343C	0.989
YBL109W	0.989
iYMR325W	0.989
iYIL177C-1	0.989
iYLR461W-0	0.989
iYOL135C	0.989
YER060w-A	0.988
YLL065W	0.988
iYAL069W	0.988
iYBL109W-0	0.988
iYGR295C-0	0.988
iYEL068C	0.9875
iYPR079W	0.9875
YAL051W	0.987
YFL063W	0.987
iYPL283C-0	0.987
iYPL283C-1	0.987
TEL9L	0.987
iYLR181C	0.987
iYNR076W	0.987
iYNL337W	0.987
iYGLWomega1-0	0.987
iYLR432W	0.987
iYKL041W	0.9865
YOL166C	0.9865
YDL102W	0.986
YFL068W	0.986
iYFL063W	0.986
iYJR161C	0.986
YPR117W	0.986
iYPL200W	0.986
iYPR201W-1	0.986
iYLL049W	0.986
YOR016C	0.985
iYDR543C	0.985
iYOL162W	0.985
iYMR012W	0.985
YAR075W	0.984
YJR029W	0.984
iYPR046W	0.984
iYNR020C	0.9835
YDR482C	0.9835
YDR162C	0.983
iYMR218C	0.983
iYDR544C	0.983
iYLL066C-1	0.983
iYNL335W	0.983
iYPL178W	0.983
YMR157C	0.983
iYLR461W-1	0.983
iYLL043W	0.983
Q0005	0.982
TEL3R	0.982
iYEL074W	0.982
iYDL030W	0.982
iYGL155W	0.9815
YHR015W	0.981
YBL079W	0.981
YJL150W	0.981
iYER041W	0.981
iYLR035C-A-0	0.981
YAR044W	0.98
YHR030C	0.98
YMR284W	0.98
YDR244W	0.98
iYML133C-1	0.98
TEL15R-1	0.98
Q0325	0.98
iYPR149W	0.98
YCLWomega1	0.979

Top 10% Ranking Targets IP'd by Rif1-proA

Feature	Rank
YKL225W	0.979
YAL069W	0.979
YDR544C	0.979
YDR485C	0.979
iYNR029C	0.978
iYHL049C-0	0.978
iYPL176C	0.978
iYOL130W	0.978
YML104C	0.9775
YCL076W	0.977
iYKL038W	0.977
iYLR166C	0.977
iYJR044C	0.977
YGL113W	0.9765
iYLR301W	0.9765
YLR384C	0.976
YNL102W	0.976
YHR027C	0.976
YER111C	0.976
YHL023C	0.976
YMR191W	0.976
iYDL091C	0.976
iYJR093C	0.976
iYML022W	0.976
YNR077C	0.976
iYLL067C-1	0.976
TEL6R	0.976
iYPR078C	0.976
iYLR144C	0.9755
iYAL043C	0.9755
iYPL132W	0.975
YLL034C	0.975
iYLL035W	0.975
itD(GUC)J4	0.9745
YKR095W	0.974
YDL212W	0.974
iYJLWdelta9	0.974
iYKL207W	0.974

Feature	Rank
YDRCTy1-2C	0.974
iYML133C-0	0.974
YHR079C-B	0.9735
YCR093W	0.973
YCLWTy5-1A	0.973
YGR018C	0.973
iYERWomega2-1	0.973
iYEL073C-4	0.973
iYCRWdelta9	0.9725
YDR484W	0.972
iYHR173C	0.972
iYFL064C	0.972
iYEL020C	0.972
YML133C	0.972
YMR107W	0.972
YJL213W	0.972
iYHR074W	0.972
iYJR046W	0.9715
iYAR035W	0.971
iYBL111C	0.971
iYHR211W	0.971
YBR301W	0.971
iYFL068W	0.97
iYOR072W-1	0.97
isnR14	0.9695
iYGL126W	0.9695
YER033C	0.969
iYPL242C	0.969
iYJR105W	0.9685
YLR098C	0.968
YGL215W	0.968
YDR170W-A	0.968
iYIL177C-0	0.968
iYGR295C-1	0.968
itE(UUC)L	0.968

Feature	Rank
Q0155	0.9675
YOR159C	0.967
iYJR092W	0.967
SNR66	0.967
iYNR040W	0.967
iYOR344C-1	0.967
iYJR111C	0.967
YGL122C	0.9665
YBL088C	0.966
iRDN37-1	0.966
YHR092C	0.965
YBL034C	0.965
iYAL068C-0	0.965
YNL338W	0.965
iYLR233C	0.965
itR(UCU)K	0.965
itM(CAU)J2	0.965
iYCR096C	0.965
iYMR247C	0.9645
YDL001W	0.9645
iYCL017C	0.964
YCRWomega3	0.964
YHR049W	0.964
iYNLCdelta1-0	0.964
iYPR041W	0.964
iYPL175W	0.964
iYIR033W	0.9635
iYPL041C	0.963
YDR147W	0.963
iYGR071C	0.963
SNR46	0.963
iYCL075W	0.963
iYNR030W	0.963
YBR136W	0.9625
CEN16	0.9625
YAL063C	0.962



Top 10% Ranking Targets IP'd by Rif1-proA

Feature	Rank
iYLR466W	0.962
iYAL039C-0	0.962
iYLR076C	0.962
YEL074W	0.962
YGRCTy1-2C	0.962
iYLL034C	0.962
iYJR115W	0.962
iYCR073W-A	0.9615
YKL083W	0.9615
YBL040C	0.961
iYKL154W	0.961
iYMR110C	0.961
iYLL050C	0.961
YLR432W	0.9605
YAR073W	0.96
iYGL261C	0.96
YDL134C-A	0.96
EMPTY	0.96
iYHL039W	0.9595
YPR042C	0.959
YCLX10C	0.959
iYML047C	0.959
YDR509W	0.959
YJR130C	0.959
iYOL127W	0.959
iYJR042W	0.959
YIL013C	0.9585
YBL005W-B	0.9585
YAR050W	0.958
iYOR393W	0.958
YEL007W	0.9575
iYIR014W	0.957
YIL176C	0.957
iYCR098C-1	0.957
YLR438C-A	0.957
itP(UGG)O2	0.957
iYCR093W	0.957

Feature	Rank
YPL253C	0.9565
iYDR330W	0.956
iYAL061W	0.955
iYCR103C	0.955
YOR394W	0.955
iYLR054C	0.9545
YPL065W	0.954
LSR1	0.954
iYDR091C	0.954
iYPLWdelta8	0.954
YAR015W	0.9535
YJL005W	0.9535
iYMR295C	0.953
YPR030W	0.953
YMR179W	0.953
iYKR056W	0.953
YLL066C	0.953
iYPL008W	0.9525
YER037W	0.9525
YMR258C	0.952
YNL212W	0.952
iYHL046C	0.952
iYLR164W	0.952
iYKL082C	0.952
iYDL114W	0.952
YOL087C	0.952
iYORWtau3	0.952
iYDL075W	0.951
YER002W	0.951
iYPL147W	0.951
iYDR285W	0.951
iYPR051W	0.951
YBR017C	0.95
YNL070W	0.95
YER190W	0.95
iYLL063C	0.95
YHL035C	0.95
YLRCdelta16	0.95

Feature	Rank
iYEL077C	0.95
iYMR232W	0.9495
iYHL014C	0.949
YLL040C	0.949
iYML057W	0.949
iYKL175W	0.949
YGR042W	0.949
iYHRCdelta14-B	0.949
iYPL191C	0.949
YOL046C	0.9485
YDR277C	0.948
YFL066C	0.948
iYNR035C	0.948
iYEL058W	0.948
iYGL041C	0.948
YOR308C	0.947
YHL028W	0.947
iYLRWdelta6-A	0.947
iYBL109W-1	0.947
iYJR101W	0.947
iYGL188C	0.9465
YER013W	0.946
iYLR102C	0.946
itN(GUU)N2	0.946
YDL015C	0.946
iYPL228W	0.946
iYLR329W	0.9455
YCR096C	0.945
YIL020C	0.945
YHR216W	0.945
iYNR026C	0.945
YCL038C	0.945
iYOL166C	0.944
YKL189W	0.944
iYCR097W	0.944
YBL047C	0.9435

Top 10% Ranking Targets IP'd by Rif1-proA

Feature	Rank
YJLCdelta6	0.9435
YGL090W	0.9435
YDR219C	0.943
iYNR006W	0.943
YHR218W	0.943
YDR499W	0.942
iYNL336W-1	0.942
YOR089C	0.942
iYLL066C-0	0.942
iYOR229W	0.942
YPRCdelta15	0.942
iYNL303W	0.942
YJR019C	0.942
iYNL165W	0.942
iYKL225W	0.942
iYNLCdelta1-2	0.942
iYNL243W	0.942
iYDL066W	0.942
iYPL265W	0.942
YBR285W	0.941
iYIL099W	0.941
itA(AGC)M2	0.941
iYLR146C	0.941
YEL077C	0.941
YDR009W	0.941
YIR010W	0.941
YOL161C	0.941
YLR461W	0.9405
iYDL160C	0.94
iYMR234W	0.94
iYOR067C	0.94
YDR182W	0.94
YDR405W	0.94
iYNR036C	0.94
YPR202W	0.94
TEL15R-6	0.94

Feature	Rank
iYGR015C	0.94
YIL125W	0.9395
iYEL011W	0.939
iYML001W	0.939
YER023W	0.939
iYJR037W	0.939
YML049C	0.9385
YPR044C	0.9385
YDR133C	0.938
YCL052C	0.938
YGR244C	0.938
Q0010	0.938
YBL085W	0.938
YJR121W	0.938
YMR167W	0.938
iYNL027W	0.937
YJR013W	0.937
Q0050	0.937
iYHRCdelta5	0.937
YER137C	0.937
iYAL020C	0.937
YGRCTy1-2A	0.936
iYAL041W	0.936
iYJR097W	0.936
iYCR104W	0.936
YML103C	0.9355
YKR096W	0.9355
YER189W	0.935
YAR074C	0.935
YDR310C	0.935
YDR007W	0.935
YGR108W	0.935
iYHR213W-0	0.935
iYGL057C	0.935
iYLR364W	0.935
iYDL206W	0.935
itV(AAC)G1	0.9345

Feature	Rank
iYFR007W	0.934
iYNR021W	0.934
YDRCTy1-2D	0.934
iYJR009C-1	0.934
YLR037C	0.934
iYJR084W	0.934
iYHL012W	0.933
YDR345C	0.933
iYLR035C-A-1	0.933
YNL103W	0.933
YFL057C	0.933
YEL006W	0.933
YOR017W	0.9325
YGR032W	0.9325
itM(CAU)J3	0.9325
iYKL085W	0.932
iYOL024W	0.9315
YER084W	0.9315
iSNR55	0.931
YHR219W	0.931
YJR115W	0.931
itE(UUC)E3	0.931
YMR075W	0.931
iYDR143C	0.9305
iYHR163W	0.93
YDR080W	0.93
YML132W	0.93
iYDR233C	0.93
iYGR262C	0.93
YER125W	0.93
iYMR267W	0.93
iYOR204W	0.9295
iYFR053C-1	0.929
YKR013W	0.929
YNL167C	0.929
YCR073C	0.929

Top 10% Ranking Targets IP'd by Rif1-proA

Feature	Rank
YMR045C	0.929
tL(GAG)G	0.9285
YIL080W	0.928
iYDL072C	0.928
iYMR179W	0.928
YNR076W	0.928
YER064C	0.928
iYAL064W-B	0.928
iYLL046C	0.928
YMR173W	0.927
YIR033W	0.927
YGL195W	0.927
iYPL282C	0.927
iYHR038W	0.927
YBL101W-B	0.927
YGRCTy1-2D	0.927
YDR466W	0.927
iYDL068W	0.927
iYIL128W	0.926
YDR308C	0.926
itD(GUC)K	0.926
YDR470C	0.926
YMR165C	0.926
YHR138C	0.926
iYDR484W	0.9255
itQ(UUG)C	0.9255
iYMR028W	0.9255
iYIL117C	0.925
iYIR043C	0.925
YJR116W	0.925
YGL077C	0.925
iYKR077W	0.925
YDR089W	0.9245
iYAL001C	0.9245
iYPL208W	0.924
iYER022W	0.924
YGL085W	0.924

Feature	Rank
iYOL042W	0.924
iYHR218W	0.924
iYMR132C	0.924
YGL062W	0.924
YGR204W	0.924
YGL261C	0.923
iYBR154C	0.923
YCR098C	0.923
iYJR008W	0.923
YPL283C	0.923
iCEN14	0.923
YIL149C	0.923
iYPL030W	0.922
iYEL051W	0.922
iYGL012W	0.922
YDR024W	0.922
YDR475C	0.922
iYJR040W	0.922
iYKR065C	0.9215
iYLR190W	0.9215
YGL086W	0.9215
iYMR225C	0.921
YCR039C	0.921
iYIR040C	0.921
iYGR085C	0.921
YML061C	0.921
iYLL067C-0	0.921
iYLR172C	0.921
YDR039C	0.921
YER167W	0.921
iYNL002C	0.921
YAR037W	0.921
YMR164C	0.921
iYLL051C	0.921
YDR311W	0.9205
YER161C	0.9205
YCL054W	0.92
itW(CCA)J	0.92

Feature	Rank
YCR083W	0.92
iYCRWomega3	0.92
iYKR050W	0.92
iYOR287C	0.92
YER082C	0.92
iYGR177C	0.92
iYIR008C	0.919
IntYBR048W	0.919
iYLR177W	0.919
YMR124W	0.919
iYGR249W-0	0.919
iYLR345W	0.919
YLR025W	0.919
iYMR119W-A	0.919
iYOR193W	0.9185
iYOL146W	0.918
iYOL149W	0.918
iYDR469W	0.918
iYBR078W	0.918
YDR077W	0.918
YCL068C	0.918
YML075C	0.918
iYPR148C-0	0.918
YBR042C	0.918
YOLWdelta6	0.918
iYAL004W	0.918
YDR486C	0.918
YCL075W	0.917
YDL010W	0.917
YCLWTy5-1C	0.917
Q0035	0.917
iYJL047C	0.917
YILCdelta5	0.916
iYDL178W	0.916
YPR036W	0.916

Top 10% Ranking Targets IP'd by Rif1-proA

Feature	Rank
YCR097W	0.916
iYHRWdelta7	0.916
iYHR057C	0.916
YCL037C	0.916
YDL248W	0.916
iYDR316W	0.915
YMR051C	0.915
iYLRCdelta5	0.915
iYOR341W	0.915
iYLR335W	0.915
iYKL198C	0.915
iYCR102W-A	0.915
YLR302C	0.9145
YKL208W	0.914
iYCR106W	0.914
iYNL261W	0.914
iYPL150W	0.914
iYLR192C	0.914
iYDL046W	0.914
YMR204C	0.9135
YDR334W	0.913
YDR275W	0.913
iYDR032C-0	0.913
YDR279W	0.913
Q0075	0.913
iYEL075C	0.913
YLR369W	0.913
iYGR014W	0.913
iYJL073W	0.913
YMR119W-A	0.913
iYMR206W	0.913
iYOR094W	0.9125
iCEN8	0.9125
iYAL063C-2	0.912
iYEL014C	0.912
iYFR027W	0.912
YGR014W	0.912
YDR115W	0.912

Feature	Rank
YMR324C	0.911
iYCR090C-1	0.911
RDN37-1C	0.911
iYOR114W	0.911
iYHL022C	0.911
iYGR236C	0.911
iYKR029C	0.911
iYLR220W	0.911
iYGL014W	0.911
YDR502C	0.911
iYKR045C	0.911
iYPL089C	0.911
iYERWdelta21-0	0.911
YBL108W	0.91
iYIR042C	0.91
iYEL030W	0.91
YNL336W	0.91
YBR038W	0.91
iYKR042W	0.91
YDR507C	0.91
YDL145C	0.91
YNL106C	0.91
iYNL040W	0.9095
YCL056C	0.909
YML100W-A	0.909
YGR241C	0.909
YER014W	0.909
YDR424C	0.909
YIL100W	0.909
YDL209C	0.909
YAR042W	0.909
iYAL008W	0.9085
YHR117W	0.908
YKR064W	0.908
iYGR265W	0.908
iYER011W	0.908
iYGL037C	0.908

Feature	Rank
iYGL122C	0.908
IntYHR010W	0.908
YCR097WA	0.908
YDR249C	0.908
YLR396C	0.9075
YKL078W	0.907
YDL002C	0.907
YMR014W	0.907
iYOR321W	0.907
YDL225W	0.907
YJR069C	0.9065
iYDR440W	0.9065
iYLL054C	0.906
iYLR162W-B	0.906
iYLR118C	0.906
YGL015C	0.906
YLR310C	0.906
YCL039W	0.906
iYDL200C	0.906
YIL144W	0.906
YDL240W	0.906
YERWdelta21	0.905
iYHL004W	0.905
YIL177C	0.905
YLR147C	0.905
YKL215C	0.905
iYBR187W	0.905
iYJL018W	0.905
iYDR235W	0.905
iYDRCdelta3	0.905
YPR078C	0.905
YAL026C	0.9045
iYPR135W	0.9045
iYIR023W	0.904
YKL224C	0.904
iYLR128W	0.904
YML130C	0.904

Top 10% Ranking Targets IP'd by Rif1-proA

Feature	Rank
YHR044C	0.904
YDRCTy1-2B	0.904
iYOR376W-0	0.904
iYDR404C	0.904
iYPL230W	0.904
YLL064C	0.904
iYFL014W	0.904
YCR097WB	0.904
iYMR053C	0.904
iYBR210W	0.903
YCL069W	0.903
iYKR014C	0.903
SNR67	0.903
iYNR032W	0.903
YKLCdelta4	0.903
iYOR179C-0	0.903
YJL155C	0.903
iYGL184C	0.9025
YKR030W	0.9025
iYAL065C	0.902
iYLR119W	0.902
iYOR205C	0.902
iYNR057C	0.902
YOLCdelta8	0.902
YER139C	0.902
iYLR278C	0.902
iCEN6	0.902

Feature	Rank
iYGR158C	0.9015
iYLL008W	0.9015
YDL193W	0.901
iYGL097W-1	0.901
YOL014W	0.901
TEL15R-2	0.901
YILWTy3-1C	0.901
YNL034W	0.901
iYPR203W	0.901
YMR043W	0.9005
YDR153C	0.9
YCR032W	0.9
YKR048C	0.9
YLR179C	0.9
iYHR059W	0.9
iYKL193C	0.9
YIL068C	0.9
YLRWdelta15	0.9
YMR231W	0.8995
YFR034C	0.899
iYPRWdelta16	0.899
YJL223C	0.899
iYDR439W-0	0.899
iYLL041C	0.899
YDR508C	0.8985
YIR044C	0.898

Feature	Rank
iYGL090W	0.898
YBLWTy2-1A	0.898
iYCR031C	0.898
iYCR038C	0.898
iYKR038C	0.898
iYMR100W	0.898
YBR302C	0.898
iYLL013C	0.898
YGR061C	0.897
YKL174C	0.8965
iYNR048W	0.8965
YLR355C	0.8965
YER034W	0.896
YDL097C	0.896
YHR177W	0.896
iYBR215W	0.8955
YKL095W	0.895
iYPL043W	0.895
iYKL144C	0.895
YFL020C	0.895
iYCRWdelta12	0.895
iYJR103W	0.895
YMR270C	0.8945

Common Targets IP'd by Both Rif1-proA (top 5%) and Rap1p (top 8%)

Feature	Rank	ORF or Intergenic	Telomeric
IYHR217C	0.999	INTERGENIC	TRUE
IYNL338W	0.999	INTERGENIC	TRUE
IYJL225C-1	0.997	INTERGENIC	TRUE
YDR545W	0.997	ORF	TRUE
IYHR216W	0.996	INTERGENIC	TRUE
IYERWOMEGA2-0	0.994	INTERGENIC	TRUE
IYNL339C	0.994	INTERGENIC	TRUE
TEL1R	0.994	INTERGENIC	TRUE
YHR217C	0.994	ORF	TRUE
IYHR219W	0.993	INTERGENIC	TRUE
YMR326C	0.993	ORF	TRUE
IYLL065W	0.992	INTERGENIC	TRUE
IYDR542W	0.991	INTERGENIC	TRUE
IYHL049C-1	0.991	INTERGENIC	TRUE
YJR162C	0.991	ORF	TRUE
IYJL225C-0	0.99	INTERGENIC	TRUE
IYIL177C-1	0.989	INTERGENIC	TRUE
IYLR461W-0	0.989	INTERGENIC	TRUE
IYMR325W	0.989	INTERGENIC	TRUE
YBL109W	0.989	ORF	TRUE
IYAL069W	0.988	INTERGENIC	TRUE
IYBL109W-0	0.988	INTERGENIC	TRUE
IYGR295C-0	0.988	INTERGENIC	TRUE
YLL065W	0.988	ORF	TRUE
IYGLWOMEGA1-0	0.987	INTERGENIC	TRUE
IYNL337W	0.987	INTERGENIC	TRUE
IYNR076W	0.987	INTERGENIC	TRUE
IYPL283C-0	0.987	INTERGENIC	TRUE
IYPL283C-1	0.987	INTERGENIC	TRUE
TEL9L	0.987	INTERGENIC	TRUE
YFL063W	0.987	ORF	TRUE
YOL166C	0.9865	ORF	TRUE
IYFL063W	0.986	INTERGENIC	TRUE
IYJR161C	0.986	INTERGENIC	TRUE
IYPR201W-1	0.986	INTERGENIC	TRUE
YFL068W	0.986	ORF	TRUE
IYDR543C	0.985	INTERGENIC	TRUE
IYOL162W	0.985	INTERGENIC	TRUE
YAR075W	0.984	ORF	TRUE
IYDR544C	0.983	INTERGENIC	TRUE

Common Targets IP'd by Both Rif1-proA (top 5%) and Rap1p (top 8%)

Feature	Rank	ORF or Intergenic	Telomeric
IYLL066C-1	0.983	INTERGENIC	TRUE
IYLR461W-1	0.983	INTERGENIC	TRUE
IYEL074W	0.982	INTERGENIC	TRUE
IYML133C-1	0.98	INTERGENIC	TRUE
TEL15R-1	0.98	INTERGENIC	TRUE
YAL069W	0.979	ORF	TRUE
YCLWOMEGA1	0.979	LTR	TRUE
YDR544C	0.979	ORF	TRUE
YKL225W	0.979	ORF	TRUE
IYHL049C-0	0.978	INTERGENIC	TRUE
YCL076W	0.977	ORF	TRUE
IYLL067C-1	0.976	INTERGENIC	TRUE
TEL6R	0.976	INTERGENIC	TRUE
IYML133C-0	0.974	INTERGENIC	TRUE
IYEL073C-4	0.973	INTERGENIC	TRUE
IYERWOMEGA2-1	0.973	INTERGENIC	TRUE
YCLWTY5-1A	0.973	TRANS	TRUE
IYFL064C	0.972	INTERGENIC	TRUE
IYBL111C	0.971	INTERGENIC	TRUE
IYGR295C-1	0.968	INTERGENIC	TRUE
IYIL177C-0	0.968	INTERGENIC	TRUE
YNL338W	0.965	ORF	TRUE
IYCL075W	0.963	INTERGENIC	TRUE
IYLR466W	0.962	INTERGENIC	TRUE
YEL074W	0.962	ORF	TRUE
YAR073W	0.96	ORF	TRUE
IYOR393W	0.958	INTERGENIC	TRUE
YIL176C	0.957	ORF	TRUE
YOR394W	0.955	ORF	TRUE
YLL066C	0.953	ORF	TRUE
IYHL046C	0.952	INTERGENIC	TRUE
YER190W	0.95	ORF	TRUE
YFL066C	0.948	ORF	TRUE
IYBL109W-1	0.947	INTERGENIC	TRUE
YHR216W	0.945	ORF	TRUE
IYPR079W	0.9875	INTERGENIC	FALSE
IYLR166C	0.977	INTERGENIC	FALSE
IYPL132W	0.975	INTERGENIC	FALSE
IYCR096C	0.965	INTERGENIC	FALSE
YCRWOMEGA3	0.964	ORF	FALSE

Common Targets IP'd by Both Rif1-proA (top 5%) and Rap1p (top 8%)

Feature	Rank	ORF or Intergenic	Telomeric
YLR432W	0.9605	ORF	FALSE
IYCR103C	0.955	INTERGENIC	FALSE
IYKR056W	0.953	INTERGENIC	FALSE
IYGL188C	0.9465	INTERGENIC	FALSE
YCR096C	0.945	ORF	FALSE

Targets IP'd by Rif1-proA (top 5%) but not Rap1p (top 8%)

Feature	Rank	Gene
YDL176W	1	
iYPL193W	1	
iYMR137C	1	
iYJL089W	0.998	
YHL027W	0.998	RIM101
YBR279W	0.997	PAF1
YPL058C	0.996	PDR12
YER011W	0.995	TIR1
YIL173W	0.995	VTH1
iYPR148C-1	0.994	
iYPL212C	0.994	
iYEL004W	0.994	
YHRCTy1-1D	0.994	
YHL039W	0.993	
iYIR010W	0.992	
YDR248C	0.9915	
YCL018W	0.991	LEU2
YPR168W	0.991	NUT2
YDR170C	0.991	SEC7
iYPR035W	0.9905	
YNL337W	0.99	
YER166W	0.99	
YGR098C	0.989	ESP1
YDR343C	0.989	HXT6
iYOL135C	0.989	
YER060w-A	0.988	FCY22
iYEL068C	0.9875	
YAL051W	0.987	YAF1
iYLR432W	0.987	
iYLR181C	0.987	
iYKL041W	0.9865	
YDL102W	0.986	CDC2
YPR117W	0.986	
iYPL200W	0.986	
iYLL049W	0.986	

Feature	Rank	Gene
YOR016C	0.985	ERP4
iYMR012W	0.985	
YJR029W	0.984	
iYPR046W	0.984	
YDR482C	0.9835	
iYNR020C	0.9835	
YDR162C	0.983	NBP2
YMR157C	0.983	
iYPL178W	0.983	
iYNL335W	0.983	
iYMR218C	0.983	
iYLL043W	0.983	
iYDL030W	0.982	
TEL3R	0.982	
Q0005	0.982	
iYGL155W	0.9815	
YHR015W	0.981	MIP6
YBL079W	0.981	NUP170
YJL150W	0.981	
iYLR035C-A-0	0.981	
iYER041W	0.981	
YAR044W	0.98	OSH1
YDR244W	0.98	PEX5
YHR030C	0.98	SLT2
YMR284W	0.98	YKU70
Q0325	0.98	
iYPR149W	0.98	
YDR485C	0.979	
iYPL176C	0.978	
iYOL130W	0.978	
iYNR029C	0.978	
YML104C	0.9775	MDM1
iYKL038W	0.977	
iYJR044C	0.977	

Targets IP'd by Rif1-proA (top 5%) but not Rap1p (top 8%)

Feature	Rank	Gene
YGL113W	0.9765	SLD3
iYLR301W	0.9765	
YNL102W	0.976	POL1
YHR027C	0.976	RPN1
YER111C	0.976	SWI4
YNR077C	0.976	
YMR191W	0.976	
YHL023C	0.976	
iYPR078C	0.976	
iYML022W	0.976	
iYJR093C	0.976	
iYDL091C	0.976	
YLR384C	0.976	IKI3
iYLR144C	0.9755	
iYAL043C	0.9755	
YLL034C	0.975	
iYLL035W	0.975	
itD(GUC)J4	0.9745	
YDRCTy1-2C	0.974	
iYKL207W	0.974	
iYJLWdelta9	0.974	
YKR095W	0.974	MLP1
YDL212W	0.974	SHR3
YHR079C-B	0.9735	IRE1
YGR018C	0.973	
YCR093W	0.973	CDC39
iYCRWdelta9	0.9725	
YMR107W	0.972	
YJL213W	0.972	
iYHR173C	0.972	
iYHR074W	0.972	
iYEL020C	0.972	
YML133C	0.972	REC114
YDR484W	0.972	SAC2
iYJR046W	0.9715	

Feature	Rank	Gene
YBR301W	0.971	
iYHR211W	0.971	
iYAR035W	0.971	
iYOR072W-1	0.97	
iYFL068W	0.97	
iYGL126W	0.9695	
isnR14	0.9695	
iYPL242C	0.969	
YER033C	0.969	ZRG8
iYJR105W	0.9685	
YDR170W-A	0.968	
itE(UUC)L	0.968	
YLR098C	0.968	CHA4
YGL215W	0.968	CLG1
Q0155	0.9675	
SNR66	0.967	
iYOR344C-1	0.967	
iYNR040W	0.967	
iYJR111C	0.967	
iYJR092W	0.967	
YOR159C	0.967	SME1
YGL122C	0.9665	NAB2
iRDN37-1	0.966	
YBL088C	0.966	TEL1
iYLR233C	0.965	
iYAL068C-0	0.965	
itR(UCU)K	0.965	
itM(CAU)J2	0.965	
YHR092C	0.965	HXT4
YBL034C	0.965	STU1
iYMR247C	0.9645	
YDL001W	0.9645	
YHR049W	0.964	
iYPR041W	0.964	
iYPL175W	0.964	

Targets IP'd by Rif1-proA (top 5%) but not Rap1p (top 8%)

Feature	Rank	Gene
iYNLCdelta1-0	0.964	
iYCL017C	0.964	
iYIR033W	0.9635	
iYPL041C	0.963	
SNR46	0.963	
iYNR030W	0.963	
iYGR071C	0.963	
YDR147W	0.963	EKI1
CEN16	0.9625	
YBR136W	0.9625	MEC1
YGRCTy1-2C	0.962	
iYLR076C	0.962	
iYLL034C	0.962	
iYJR115W	0.962	
iYAL039C-0	0.962	
YAL063C	0.962	FLO9
YKL083W	0.9615	
iYCR073W-A	0.9615	
iYMR110C	0.961	
iYLL050C	0.961	
iYKL154W	0.961	
YBL040C	0.961	ERD2
YDL134C-A	0.96	
iYGL261C	0.96	
iYHL039W	0.9595	
YPR042C	0.959	PUF2
YDR509W	0.959	
YCLX10C	0.959	
iYOL127W	0.959	
iYML047C	0.959	
iYJR042W	0.959	
YJR130C	0.959	STR2
YBL005W-B	0.9585	
YIL013C	0.9585	PDR11

Feature	Rank	Gene
YAR050W	0.958	FLO1
YEL007W	0.9575	TOS9
YLR438C-A	0.957	
iYIR014W	0.957	
iYCR098C-1	0.957	
iYCR093W	0.957	
itP(UGG)O2	0.957	
YPL253C	0.9565	VIK1
iYDR330W	0.956	
iYAL061W	0.955	
iYLR054C	0.9545	
LSR1	0.954	
iYPLWdelta8	0.954	
iYDR091C	0.954	
YPL065W	0.954	VPS28
YAR015W	0.9535	ADE1
YJL005W	0.9535	CYR1
iYMR295C	0.953	
YPR030W	0.953	CSR2
YMR179W	0.953	SPT21
iYPL008W	0.9525	
YER037W	0.9525	PHM8
YOL087C	0.952	
YNL212W	0.952	
YMR258C	0.952	
iYORWtau3	0.952	
iYLR164W	0.952	
iYKL082C	0.952	
iYDL114W	0.952	
YER002W	0.951	
iYPR051W	0.951	
iYPL147W	0.951	
iYDR285W	0.951	
iYDL075W	0.951	
YLRCdelta16	0.95	

Targets IP'd by Rif1-proA (top 5%) but not Rap1p (top 8%)

Feature	Rank	Gene
YHL035C	0.95	
iYLL063C	0.95	
iYEL077C	0.95	
YBR017C	0.95	KAP104
YNL070W	0.95	TOM7
iYMR232W	0.9495	
iYHL014C	0.949	
YGR042W	0.949	
iYPL191C	0.949	
iYML057W	0.949	
iYKL175W	0.949	
iYHRCdelta14-B	0.949	
YLL040C	0.949	VPS13
YOL046C	0.9485	
iYNR035C	0.948	
iYGL041C	0.948	

Feature	Rank	Gene
iYEL058W	0.948	
YDR277C	0.948	MTH1
iYLRWdelta6-A	0.947	
iYJR101W	0.947	
YOR308C	0.947	SNU66
YHL028W	0.947	WSC4
YDL015C	0.946	
iYPL228W	0.946	
iYLR102C	0.946	
itN(GUU)N2	0.946	
YER013W	0.946	PRP22
iYLR329W	0.9455	
iYNR026C	0.945	
YCL038C	0.945	AUT4
YIL020C	0.945	HIS6

Chapter 4

Perspective
Conclusions
Future Directions

Too many pieces of music finish too long after the end.
Igor Stravinsky (1882 - 1971)

Perspective

When I joined the Blackburn lab discoveries were being made at a rapid pace and redefining our notions of how telomeres functioned. The theory that telomeres acted as a mitotic clock that contributed to cellular aging was a wildly popular and well supported in a number of model systems and cell types. It was increasingly being appreciated that cells might undergo senescence for a reason: after their "useful" lifespan, cells that had acquired errors might be *prevented* from becoming cancerous by limiting their replicative potential. Also around this time, a growing body of evidence correlating increased telomerase activity with cancerous growth shifted the focus of study toward the relationships between telomerase, telomeres and genomic stability. I was interested in how telomere length regulation affected the links between telomere maintenance and genomic instability.

While considerable work had been done to study the effects of telomere shortening on growth rate, genome stability, and cellular viability, the effects of telomere *lengthening* remained largely unstudied. Telomere lengthening was not as well studied, in part, for technical reasons: there were few effective ways to overly elongate telomeric DNA in living cells until genes for the repressive chromatin components were cloned and mutated or knocked out. In yeasts, the *RAP1* gene performs this function, and mutants with C-terminal truncations exhibit significant telomere elongation, deregulation, growth defects, and chromosome loss (Kyrion, Boakye et al. 1992). Once the hTRF genes had been cloned and dominant negative alleles introduced into mammalian cells, the detrimental effects of elongated, deregulated telomeres in mammalian systems were more evident. These telomeres

could create end-to-end fusions and genomic instability (van Steensel, Smogorzewska et al. 1998). I was very interested in how the loss of length regulation could affect chromosome stability and I chose to study this problem in budding yeasts.

Telomere Deregulation, Not Elongation Results in Genomic Instability in Yeast

At the time I began my thesis work, there was a growing list of factors that affected telomere length regulation in *S. cerevisiae* (See Chapter 1, Table 1), but very few ways to study elongated/deregulated telomeres without dramatically perturbing the telomeric chromatin. It was known that Rap1p interacted with Sir3p (Palladino, Laroche et al. 1993; Moretti, Freeman et al. 1994), Sir4p (Palladino, Laroche et al. 1993; Moretti, Freeman et al. 1994), and Rif1p (Hardy, Sussel et al. 1992), but Rif2p had not yet been identified. The available alleles of RAP1 that resulted in telomere lengthening and deregulation were C-terminal truncations that also lacked the ability to bind to any of its known binding partners (Kyrion, Boakye et al. 1992), making it difficult to study the mutual interactions between components of the telomeric chromatin complex. The *RAP1* gene had been identified in *K. lactis*, which had the advantage of many *TER1* template mutants that caused telomere length phenotypes (McEachern and Blackburn 1995). This panoply of template mutants was predicted to have diverse effects on klRap1p binding and it had already been shown that elongation/deregulation caused by some of these mutants was not dependent on *in vitro* klRap1p binding (Krauskopf and Blackburn 1998).

In most systems where it was possible to experimentally perturb the telomeric chromatin, the result was generally described as "elongation" or "loss of length control". The distinction between telomere length and the ability to *regulate* telomeres at any

length was not one that the field had addressed in detail. A newly developed procedure made it possible to distinguish between the effects of extreme telomere elongation or telomere deregulation: the reintroduction of the phenotypically silent, functionally WT *ter1-Bcl* allele into strains that contained elongated/deregulated telomeric tracts "froze" the elongated telomeres at their long length, but allowed the telomeres to be re-regulated at the increased size. This tool made it possible to address whether it was the extreme telomere length itself or the lack of ability to regulate defined telomere lengths that was correlated with the apparent morphological aberrations, segregation defects, and increased DNA content of cells with mutant telomeres. Thus, one of the primary objectives of the work described in Chapter 2 was to determine which aspect of mutant telomeres was responsible for the phenotypes observed: extreme lengthening or the loss of length regulation.

The major conclusion of the work in *K. lactis* that is described in Chapter 2 was that the loss of the ability to regulate length, not the elongated nature of mutant telomeres, is detrimental to cells. As yet unpublished work from Michael McEachern in the lab had already established that re-capping rescued the sick colony phenotypes often associated with elongated telomeres, but he had not undertaken the extensive phenotypic characterization that conclusively showed that WT and re-capped, mutant *ter1* strains were phenotypically almost indistinguishable. Similarly, work by Anat Krauskopf had established that the klRap1p interaction with the most terminal repeats was essential for chromosome stability. This work and mine firmly established the terminal few telomeric repeats in *K. lactis* as a major contributor to telomere stability and had the

ability to reverse the severe growth defects, aberrant cellular morphologies, and apparent DNA segregation defects.

These results were quite unexpected. The telomeres in the *ter1-Acc*, *ter1-Bgl*, and *ter1-Kpn* mutants I worked with had telomeres up to 25 kB in length, 100 times the length of WT telomeres. The *ter1-Acc* mutants could not significantly bind klRap1p *in vitro* and yet the addition of literally 3-4 WT-like repeats allowed total re-regulation of the telomeres at this long length, even with extensive mutant tracts still existing all the way out to the chromosome end! This suggested not only that the very end repeats played a special role in stability, but also that they somehow could form a continuous, stable structure with the internal WT repeats located kilobases away at the inner end of the telomeric repeat tracts. Furthermore, the intervening *Acc* repeats that could not be bound by klRap1p were apparently "ignored". The same effect was seen with the *Bgl* and *Kpn* repeats; however, the delay before runaway elongation abruptly occurred was hundreds of generations. Also, both *Bgl* and *Kpn* repeats were predicted to bind klRap1p with the same, or slightly greater *in vitro* affinity, than WT repeats. Although these telomeres could still theoretically form a continuous stretch of telomeric chromatin, they still lacked the ability to retain length regulation. In appendix 1, I addressed whether differences in simple DNA bending might be responsible for the delayed response seen in *ter1-Bgl* or *ter1-Kpn* strains. While the DNA bend of a single *Bgl* or *Kpn* repeat was indistinguishable from WT (82-92°), this does not rule out the possibility that some other difference in DNA topology is responsible for the delayed lengthening phenotype. One possibility that we explored was that the *ter1-Bgl* and *ter1-Kpn* mutants actually titrated away all of the Rap1p and associated factors in cells,

creating a situation where it was impossible for enough klRap1p to bind the chromosome end to re-regulate it. However, the fact that re-capped *ter1-Bgl* and *ter1-Kpn* mutants, which still had dramatically elongated telomeres, were healthy suggested that this was not the case. Perhaps the existing levels of klRap1p were sufficient to coat these elongated telomeres or levels of klRap1p were upregulated to compensate for the elongated telomeric tracts.

Internal Telomeric Repeats and Distal Repeats Are Measured Differently

At first, the capping end-effect that was described by McEachern, Krauskopf, and myself was interpreted to be unique to *K lactis*. Work done by Stephan Marcand and David Shore at the time showed that the number of scRap1p C-termini at the inner end of a telomeric tract at a chromosome end "measured" telomere length (Marcand, Gilson et al. 1997). While these experiments did not address the mechanism of the "length-ruler" or how the length of the ruler itself was established or maintained, these results had the effect of creating two opposing views on length maintenance in the telomere field. The *K. lactis* capping results were considered to be a phenomenon unique to the mutant repeats of *K. lactis*. In fact, the existence of the length ruler and the terminal capping effects were not mutually exclusive and recent evidence form the Shore lab has shown that, as with *K. lactis*, the most distal repeats are, in fact, measured differently than the internal ones (Grossi, Bianchi et al. 2001). There is clearly a requirement for some minimal amount of a Rap1-nucleated complex at the chromosome end to tether factors that inhibit recombination and mask the end so that it is not treated as a double strand break. Telomeres without a Rap1-nucleated complex of some minimal size are much more prone to recombination, fusion, and loss. However, since

the terminal double stranded telomeric repeats are also, by definition, at the interface where the single stranded telomeric DNA begins, if follows that these repeats might be under different regulation. There are likely to be other subtle differences in the chromatin at this junction where the Rap1p-repressive complex must transition to the recruitment complex for telomerase. Indeed, although the mechanism remains unclear, there are intriguing results that Rap1p can actually enhance telomere formation in yeast (Ray and Runge 1998).

Senescent Cells and *ter1* Monsters: Similarities and Differences

There were a number of intriguing similarities I found between *K. lactis* strains that were in the process of senescing and those that had *ter1* template mutations. During the submission of the manuscript that comprises the text of Chapter 2, one of the reviewers commented that the phenotypes we observed might be a subpopulation of cells that actually had shortened telomeres rather than the result of deregulation. By Southern blot analysis it was evident that there existed a broad range of telomere lengths ranging from much shorter than wild type to approximately 100 times the length of wild type (Chapter 2, Figure 2). In response to this concern, I undertook the phenotypic characterization of senescing cells that is described in appendix 2. The "monster" cells that resulted from deregulated telomeres appeared somewhat different from the morphologically aberrant ones observed in senescent cultures. The major difference observed was that the cell walls of senescent cells became progressively more degraded looking and "wrinkled" as the population aged. Also, the morphologically aberrant cells were generally larger in strains with template mutations compared to *Δter1* survivors and they comprised a larger percentage of the population that was relatively constant

over long periods. That is, monster cells from *ter1-Acc*, *ter1-Bgl*, or *ter1-Kpn* strains consistently accounted for 10-15% of the population (Chapter 2, Table 1), while monster cells in senescent cultures never exceeded 5% of the population (Appendix 2, Figures 1 and 2). The percentage of the population that exhibited monster cell phenotypes in senescent and post-senescent cultures fluctuated depending upon the length status of the telomeres. Generally, only cultures with critically short telomeres had sick looking cells. Based on these differences, we argued that these monster cell population were distinct from one another.

However, it was difficult to ignore the similarities that existed between the monster cells from these two populations. The most notable similarity was that the DNA content phenotypes of both types of cells were similar: both senescent cultures and cultures with deregulated, mutant telomeres exhibited a decrease in 1N DNA content and a simultaneous increase in the 2N and greater than 2N DNA content (Chapter 2, Figure 3 and Appendix 2, Figure 2). This DNA content phenotype also disappeared from both cultures when a functionally wild-type telomerase was re-transformed in and the telomeres were re-capped (Chapter 2, Figure 3 and Appendix 2, Figure 3). Although the multi-budded morphologies were also similar between senescent cells and those with deregulated, mutant sequence telomere, monster cells in the latter strains were generally much larger.

Recent microarray evidence from Shivani Nautiyal in the lab has shown that *S. cerevisiae* cells undergoing senescence transcriptionally upregulate genes involved in mitochondrial synthesis. Supporting this result is the observation that there is a dramatic increase in the amount of mitochondria in the cell, as determined by COX4-

GFP immunofluorescence (Shivani Nautiyal, personal communication). Furthermore, the mitochondria are often morphologically quite different from WT. It is possible that the increased DNA content seen in monstrous *K. lactis* cells from strains containing template mutants is due to mitochondrial proliferation. It would be quite interesting, and technically simple, to measure the amount of mitochondria in monster cells from *ter1* template mutant strains using a similar COX4-GFP system.

The proliferation of mitochondria raises the possibility that the increased DNA content seen in both senescing and *ter1* strains is due to an increase in the amount of mtDNA and not chromosome missegregation. One argument against this possibility comes from data where the segregation of a specially marked chromosome IV is measured in *S. cerevisiae* strains containing an uncapping telomerase much like those I studies in *K. lactis*. Using a *ter1-476A* strains with chromosome IV marked by 256 tandem repeats of the Lac-operator at the telomere, Dana Smith has shown that 25% percent of cells fail to correctly segregate chromosome IV (Dana Smith, personal communication). This suggests that deregulated telomeres in strains similar to the *ter1* mutants examined in Chapter 2 do, in fact, lead specifically to segregation defects.

Perhaps the DNA content defects in senescing cells are distinct from those in strains with deregulated telomeres. It is also possible that these two types of telomere dysfunctions result in similar downstream responses. An increasing body of evidence suggests that yeast cells may undergo a form of programmed cell death (Frohlich and Madeo 2000). It may be that the similarities between senescent and deregulated *ter1* strains are an indication of how morphologically aberrant yeast cells self-destruct in the

face of chromosomal instability resulting from either shortened or deregulated telomeres.

The Protein Composition of Telomeric Chromatin over the Cell Cycle

After I and others had shown how the addition of a few functionally WT repeats to the end of an elongated, deregulated telomere allowed that telomere to become re-regulated and cells to grow without defect, I became interested in the chromatin components of that new cap and how they lent stability to the still elongated, but re-regulated telomere end. Strikingly, a small cap of only 3-4 WT-like repeats was enough to lend stability to the very elongated telomeres, largely comprised of mutant *Acc* repeats that could not bind Rap1p.

I became very interested in how such a small cap could lend stability. However, very few of the telomere proteins identified in *S. cerevisiae* had homologs cloned in *K. lactis*. Thus, I decided to change model systems and explore the role of telomeric chromatin in length regulation in *S. cerevisiae*. By the time I began the work leading to Chapter 3 of this thesis, there were dozens of proteins that affected length regulation that had been cloned in *S. cerevisiae*, but very little understanding of all the potential interactions between these factors and their mechanistic roles in telomere biology. Results were just showing that telomere replication was linked to conventional replication and the DNA damage response. A growing body of evidence suggested that telomeres had many different "states" that might be comprised of distinct protein subsets. Results from Martin and Gasser showed that the yKu, Rap1p, Sir3p, and Sir4p relocalized to sites of induced double stranded DNA damage (Martin, Laroche et al.

1999; Mills, Sinclair et al. 1999). Indeed, even during a normal cell cycle Rap1p, Sir3p, and Sir4p are displaced from chromosome ends at G2/M (Laroche, Martin et al. 2000). The human homolog of *yXRS2*, NBS1, associates specifically with S-phase telomeres (Wu, Lee et al. 2000; Zhu, Kuster et al. 2000). The single stranded G-rich overhangs in yeast are also specific to S-phase (Wellinger, Wolf et al. 1993). Work from the Zakian and other labs has shown that mutations in some replication proteins also had an effect on telomere biology (Schulz and Zakian 1994; Adams and Holm 1996; Qi and Zakian 2000; Zhou, Monson et al. 2000). To me, it followed that the repressive telomeric chromatin might look different in different states of the cell cycle. For example, a telomere being replicated was expected to have telomerase and replication factors, while prior to or after replication one might expect to see more repressive chromatin. While it was vaguely appreciated that there are probably too many factors to all be present simultaneously at the telomere, the task of sorting out which factors were simultaneously bound or mutually exclusive of one another had not been undertaken. I thought that since telomeres needed to be periodically replenished, that looking for telomeric chromatin remodeling over the course of the cell cycle might illustrate differences in the factors attached to the chromosome end. I decided to study the major negative regulators of telomere length, Rap1p and the Rif proteins, as well as the major positive factor in telomere lengthening, the telomerase itself, Est2p.

The results of the experiments described in Chapter 3 strongly indicate that the negative telomeric chromatin, minimally composed of Rap1p, Rif1p, and Rif2 is dynamically remodeled during the cell cycle. Additionally, the telomerase protein Est2p appears to remain telomere associated through all of the cell cycle except mitosis, where

it either becomes inaccessible to antibody or disassociates from the chromosome end. Alternate analysis of Est2p chromatin association data suggests that Est2p may be increasingly recruited during S-phase and displaced in G2. The presence of Est2p in G1 phase is notable, since it has been shown that cells arrested at this stage are incapable of adding telomeric DNA *in vivo* (Diede and Gottschling 1999). This suggests that telomerase may require accessory factors such as Cdc13p, Est1p, Est3p, or perhaps the replication machinery in order to begin replicating telomeric DNA in mid-late S-phase.

Rif1p Is Associates to a Broad Region of the Chromosome End

Microarray determination of the genome-wide binding sites for the Rif1 protein suggests that it associates with chromosome end regions for a distance of roughly 7 kB in from the termini, much like the Sir proteins and Rap1p (Lieb, Liu et al. 2001). This implies that Rif1 is spread along the silent chromatin, much further in than the distal telomeric tracts than had been postulated in earlier models (Wotton and Shore 1997). Furthermore, there was an end-specific difference in association of proteins to different chromosome ends: the association of Rif1p, Rap1p, and the Sir proteins extended in twice as far on Y' telomeres compared to X- telomeres. The distance of association from the end for these factors is highly relevant to models of how telomeric chromatin compacts *in vivo*. The broad association observed for Rif1p suggests that telomeric DNA is capable of associating with nucleosomal chromatin much farther in than had been supposed. This is consistent with models where the chromosome end folds over to interact with more interior regions. These associations may act like the T-loops observed in mammalian systems *in vitro*, and which are thought to protect the very end

of the chromosome from unwanted telomerase addition or recombination (Griffith, Comeau et al. 1999).

Rif2p is Modified Through the Cell Cycle

The appearance of a Rif2p doublet that changes over the cell cycle was one of the most exciting results I found in these studies. It was one of the first examples in telomere biology where a protein modification might affect the structure or function of a protein important in length regulation. Already, the phosphorylation state of Rap1p has been shown to affect its DNA binding (Tsang, Henry et al. 1990). More recent evidence has shown that the acetylation of histone H4 tails reduces it ability to interact with Sir3p (Carmen, Milne et al. 2001). These results support the idea that protein modification may play an important role in telomeric chromatin remodeling. The regulated modification of proteins involved in telomeric chromatin may be important in "melting" the interactions between telomeres and nucleosomal chromatin, or may be important in displacing components that occlude or repress telomere elongation. Although we could not determine the nature of the Rif2p modification, further study of the regulation of this doublet will doubtlessly prove interesting.

Taken together, these findings suggest that repressive telomeric chromatin is dynamically remodeled over the cell cycle and that windows of opportunity for telomerase addition appear to be largely exclusive of times when these Rap1p, Rif1p, and Rif2p proteins are maximally associated. Importantly, telomerase remains associated at most times in the cell cycle and it is merely its ability to add telomeric DNA to the chromosome end appears to be modulated. This may indicate the requirement for special accessory factors or replication fork migration to the

chromosome ends to coordinate telomeric and regular DNA synthesis. Importantly, these experiments provided preliminary evidence that protein modification may be important in the regulate processes that open and close telomeric chromatin during the cell cycle.

Future Directions

K. lactis

There are a plethora of experiments that could follow from these studies in both the *K. lactis* and the *S. cerevisiae* model systems. The identification of numerous homologs in 13 of the hemiascomycetous yeast species offers to potentially revitalize the study of telomere length maintenance in *K. lactis* (Souciet, Aigle et al. 2000). Random sequence tagging and mapping of homologous regions has already led to the identification of 1 *RIF2* homolog and 3 *RIF1* homologs, to name only a few. Cloning of full-length genes for these and other telomere related factors, coupled with the wide array of template mutants available in *K. lactis,* offers an unprecedented combination of mutations to probe telomeric chromatin. Another interesting avenue of research stemming from these data is a systematic comparison of telomere related genes between these species. This type of meta-analysis would surely indicate many of the conserved domains, folds, modification sites, and interaction faces between the telomeric proteins of these different species. Along the same lines, a reexamination of the growing bank of CDC mutants generated and characterized by Linda Silveira offers the possibility to obtain homologs of existing proteins in telomere biology as well as new genes involved in cell cycle control that affect telomere regulation. It is well worth noting that none of

the screens that have been used to identify template mutations or proteins that affect telomere length homeostasis has been done to saturation. Surely there exist other factors critical for telomere length maintenance.

There are other obvious experiments to complete in the *K. lactis* model system. Further probing of the putative topological differences between *Bgl* and *Kpn* repeats is an issue that remains to be resolved. This might also be more broadly informative about the DNA bending requirements for Rap1p in telomere length maintenance. The development of new DNA bending assays, such as pBEND2, which measures both the bend and the "twist" of the DNA offer much higher resolution. Since the time when I initially carried out the DNA bending experiments the crystal structure of the Rap1p DNA binding domain has been solved (Konig, Giraldo et al. 1996). With the crystallization conditions known it would be relatively easy to co-crystallize klRap1p with various template repeats to directly address DNA topology differences between these mutants.

S. cerevisiae

There are numerous technically feasible experiments that would further define the nature and regulation of the telomeric complex in *S. cerevisiae*. An expansion of the approach I used to identify the composition of telomere proteins over the cell cycle would greatly help to expand our understanding of telomere chromatin. Chromatin immunoprecipitation (ChIP) analysis of factors such as Sirs, Ku, Est1p, Est3p and Cdc13p, and higher resolution ChIP of Rap1p and the Rifs, would help define a WT state of telomere chromatin remodeling. This approach, coupled with the extensive mutant alleles in virtually every telomere related protein, would doubtless give the field

a very clear view of how telomeres are built, remodeled, and regulated over the cell cycle and in response to stimuli such as double stranded breaks or other conditions leading to cell cycle arrests. In particular, the mutagenesis of conserved phosphorylation, acetylation, or other modification sites would greatly inform our understanding of the role of protein modifications in telomere length regulation.

Other interesting avenues of study in *S. cerevisiae* include transcriptional analysis of telomere related gene on microarrays. It would be of great interest to know of the Rif proteins, which have been shown to affect silencing in the cell, are transcriptional modifiers of other genes and if so what their binding partners may be. My preliminary results from the genome-wide identification of Rif1p binding sites suggest that Rif1p may bind to another protein at internal genomic sites. Coupling of my microarray ChIP data with transcriptional analyses may elucidate the functions of RIFs in living cells.

The isolation and characterization of the Rif2p doublet offers to open an exciting new avenue in telomere biology, that of protein modification. While it is already known that Rap1p is extensively phosphorylated, it is unclear which phosphorylation states are important for transcriptional functions, versus telomere regulation functions. Perhaps phosphorylation affects Rap1p's binding to promoters or telomeres or its interactions with the Rifs or Sirs. Likewise, the modification of Rif2 appears to be correlated with it's association to DNA. The determination of the nature of the Rif2 modification and how it affects its function in the cell will be of great interest. Analysis of protein modifications through the cell cycle offers to uncover the molecular mechanisms of

telomeric chromatin remodeling and will very likely revolutionize our understanding of the dynamic processes involved in telomere length homeostasis.

In Conclusion

The work encompassed by the experiments in this thesis and the models derived form them help to illustrate and support a few basic principles important in telomere biology. First, the ability to regulate telomere length, and not the absolute length of telomeres, is the important criterion for preventing genomic instability. Although telomeres must be maintained at a minimal length to remain functional, even grossly elongated mutant telomeres were able to protect chromosome ends when capped with a few wild type repeats in *K. lactis*. Morphologically, cells lacking telomerase were similar but distinguishable from those containing *ter1* template mutations.

My ChIP results suggest that telomere chromatin is actively remodeled over the course of the cell cycle and that Est2p remains largely associated to chromosome ends throughout the cell cycle, except in M phase. My results also show that Rif1p is spread over the terminal 6-7 kB of the chromosome end, like Rap1p and the Sir proteins and that Rif2p is modified over the cell cycle. In conclusion, I believe that the work that I have completed in the Blackburn lab has contributed to our understanding of how telomere regulation is linked to genomic stability and the importance of chromatin remodeling in the proper maintenance of telomere length control.

In the end, everything is a gag.
Charlie Chaplin (1889 - 1977)

Don't let it end like this. Tell them I said something.
Pancho Villa, (1877 - 1923), last words

References

Adams, A. K. and C. Holm (1996). "Specific DNA replication mutations affect telomere length in Saccharomyces cerevisiae." Mol Cell Biol **16**(9): 4614-20.

Carmen, A. A., L. Milne, et al. (2001). "Acetylation of the yeast histone H4 N-terminus regulates its binding to heterochromatin protein SIR3." J Biol Chem **19**: 19.

Diede, S. J. and D. E. Gottschling (1999). "Telomerase-mediated telomere addition in vivo requires DNA primase and DNA polymerases alpha and delta." Cell **99**(7): 723-33.

Frohlich, K. U. and F. Madeo (2000). "Apoptosis in yeast--a monocellular organism exhibits altruistic behaviour." FEBS Lett **473**(1): 6-9.

Griffith, J. D., L. Comeau, et al. (1999). "Mammalian telomeres end in a large duplex loop." Cell **97**(4): 503-14.

Grossi, S., A. Bianchi, et al. (2001). "Telomere formation by rap1p binding site arrays reveals end-specific length regulation requirements and active telomeric recombination." Mol Cell Biol **21**(23): 8117-28.

Hardy, C. F., L. Sussel, et al. (1992). "A RAP1-interacting protein involved in transcriptional silencing and telomere length regulation." Genes Dev **6**(5): 801-14.

Konig, P., R. Giraldo, et al. (1996). "The crystal structure of the DNA-binding domain of yeast RAP1 in complex with telomeric DNA." Cell **85**(1): 125-36.

Krauskopf, A. and E. H. Blackburn (1998). "Rap1 protein regulates telomere turnover in yeast." Proc Natl Acad Sci U S A **95**(21): 12486-91.

Kyrion, G., K. A. Boakye, et al. (1992). "C-terminal truncation of RAP1 results in the deregulation of telomere size, stability, and function in Saccharomyces cerevisiae." Mol Cell Biol **12**(11): 5159-73.

Laroche, T., S. G. Martin, et al. (2000). "The dynamics of yeast telomeres and silencing proteins through the cell cycle." J Struct Biol **129**(2-3): 159-74.

Lieb, J. D., X. Liu, et al. (2001). "Promoter-specific binding of Rap1 revealed by genome-wide maps of protein-DNA association." Nat Genet **28**(4): 327-34.

Marcand, S., E. Gilson, et al. (1997). "A protein-counting mechanism for telomere length regulation in yeast." Science **275**(5302): 986-90.

Martin, S. G., T. Laroche, et al. (1999). "Relocalization of telomeric Ku and SIR proteins in response to DNA strand breaks in yeast." Cell **97**(5): 621-33.

McEachern, M. J. and E. H. Blackburn (1995). "Runaway telomere elongation caused by telomerase RNA gene mutations." Nature **376**(6539): 403-9.

Mills, K. D., D. A. Sinclair, et al. (1999). "MEC1-dependent redistribution of the Sir3 silencing protein from telomeres to DNA double-strand breaks." Cell **97**(5): 609-20.

Moretti, P., K. Freeman, et al. (1994). "Evidence that a complex of SIR proteins interacts with the silencer and telomere-binding protein RAP1." Genes Dev **8**(19): 2257-69.

Palladino, F., T. Laroche, et al. (1993). "SIR3 and SIR4 proteins are required for the positioning and integrity of yeast telomeres." Cell **75**(3): 543-55.

Qi, H. and V. A. Zakian (2000). "The Saccharomyces telomere-binding protein Cdc13p interacts with both the catalytic subunit of DNA polymerase alpha and the telomerase- associated est1 protein." Genes Dev **14**(14): 1777-88.

Ray, A. and K. W. Runge (1998). "The C terminus of the major yeast telomere binding protein Rap1p enhances telomere formation." Mol Cell Biol **18**(3): 1284-95.

Schulz, V. P. and V. A. Zakian (1994). "The saccharomyces PIF1 DNA helicase inhibits telomere elongation and de novo telomere formation." Cell **76**(1): 145-55.

Souciet, J., M. Aigle, et al. (2000). "Genomic exploration of the hemiascomycetous yeasts: 1. A set of yeast species for molecular evolution studies." FEBS Lett **487**(1): 3-12.

Tsang, J. S., Y. A. Henry, et al. (1990). "Phosphorylation influences the binding of the yeast RAP1 protein to the upstream activating sequence of the PGK gene." Nucleic Acids Res **18**(24): 7331-7.

van Steensel, B., A. Smogorzewska, et al. (1998). "TRF2 protects human telomeres from end-to-end fusions." Cell **92**(3): 401-13.

Wellinger, R. J., A. J. Wolf, et al. (1993). "Saccharomyces telomeres acquire single-strand TG1-3 tails late in S phase." Cell **72**(1): 51-60.

Wotton, D. and D. Shore (1997). "A novel Rap1p-interacting factor, Rif2p, cooperates with Rif1p to regulate telomere length in Saccharomyces cerevisiae." Genes Dev **11**(6): 748-60.

Wu, G., W. H. Lee, et al. (2000). "NBS1 and TRF1 colocalize at promyelocytic leukemia bodies during late S/G2 phases in immortalized telomerase-negative cells. Implication of NBS1 in alternative lengthening of telomeres." J Biol Chem **275**(39): 30618-22.

Zhou, J., E. K. Monson, et al. (2000). "Pif1p helicase, a catalytic inhibitor of telomerase in yeast." Science **289**(5480): 771-4.

Zhu, X. D., B. Kuster, et al. (2000). "Cell-cycle-regulated association of RAD50/MRE11/NBS1 with TRF2 and human telomeres." Nat Genet **25**(3): 347-52.

Appendix 4

CD Supplement

APPENDIX A

CD SUPPLEMENT

CD Supplement File List

Chapter 1

Chapter 1-Table 1-Telomere Genes.xls
Telomere Gene Table.txt

Chapter 2

Journal of Cell Ciology PDF
Smith & Blackburn JCB vol145(2) 203-14.pdf

Chapter 3

Chapter 3 Analyses

ChIPs Dotblot Analyses.xls
RIF1 Median Rank Analyses.xls
RIF1,RAP1,SIR length analysis.xls

Chapter 3 Supplemental Data

Chapter 3 Supplemental Data C-F.xls
Supplemental Data C – IDE Measurements.txt
IDE Measurement Summary.txt

Large Scale Data Sets

Ito Global 2 Hybrid Data

Ito Global 2 Hybrid.xls
Ito Global 2 Hybrid.txt

Large Scale Data Sets (cont'd)

Lieb RAP1 & SIR Annotation

Lieb_RAP1_map.pdf
Percent Distubs_rap&sirs.txt
PMotif_score_vs_tile_RNK.txt
RAP_SIR_Tel_binding.txt
RAP1_Selected_spots.txt
Sir2 map.pdf
Sir3 map.pdf
Sir4 map.pdf

Other Yeast Homologs

Various Files from Genolevures Annotation

Photo Archive

Bay Area
Blackburn Lab
Camping
Friends
World
9-11

Installers

Adobe Acrobat 5 Installers

PC Acrobat 5.exe
Mac Acrobat.bin

Acdsee Image Viewer Installers

PC acdsee.exe
Mac acdsee.hqx