UNDERSTANDING THE GENETIC REQUIREMENTS FOR THE LOSS OF END PROTECTION IN THE YEAST *KLUYVEROMYCES LACTIS*

by

SHILPA IYER

(Under the Direction of Michael McEachern)

ABSTRACT

Telomeres are the DNA-protein caps that protect chromosome ends from being repaired by either homologous recombination (HR) or non-homologous end joining (NHEJ). The proper functioning of telomeres has been linked to helping prevent cancer and aging in humans. While the telomeres of most cells are maintained by the enzyme telomerase, some cells, including 5-10% of human cancers, utilize an alternate pathway to lengthen (ALT) their telomeres, thought to involve recombination. I have discovered that a mutation (*stn1-M1*), in a gene encoding a telomere binding protein in the yeast *Kluyveromyces lactis*, leads to elongated telomeres by engaging the recombinational telomere elongation (RTE) pathway. This mutant displays many characteristics similar to human ALT cells including long telomeres, abnormal growth, chronic telomeric capping defects, and extrachromosomal telomeric circles (t-circles) making it a useful model system for understanding ALT cells. In addition, we provide evidence for another consequence of a telomere-capping defect that lead to telomere fusions and are due to NHEJ. We demonstrate that the NHEJ proteins Mre11, Rad50, Ku80 and Ligase 4 have multiple roles at *K. lactis* telomere maintenance. Most notably, we saw for the first time that Ligase 4 contributes to protecting telomeres from HR. Thus, work from this study lends further support to the

hypothesis that the capping function of telomeres is essential to prevent two major DNA repair pathways, HR and NHEJ, from acting at telomeres.

INDEX WORDS: yeast, *Kluyveromyces lactis*, telomere, ALT, recombination, non-homologous end joining, capping, DNA repair, cancer.

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DEDICATION

To my family-

My parents for their passion and intelligence,

And my husband, for his love and perseverance;

To Billu, an unruly delight, in those good old days,

And Manu-Nisha, so precious, in their own individual ways;

For I have learnt so much from all of you,

And also adapted to incorporate changes anew.

This idiosyncrasy spills from another 'ganjigunte hattamari',

Who will always remain your very own 'cheelu maree'.

ACKNOWLEDGEMENTS

The idea of education has been described in the ancient Vedic texts, as a major part of one's life spent under the tutelage of a guru or mentor, where one spends a considerable amount of time acquiring knowledge and wisdom gained through life's experiences and teachings shared by the guru. This ancient style, aims to teach the 'shishyas' or disciples *how* to think, before they march forth in quest of their own destiny. I believe that in modern times, faculty advisors play the synonymous role of mentors by taking time to understand the student's needs and helps them develop into a self motivated and successful professional.

In this regard, my advisor Michael McEachern has fulfilled both the ancient and the modern descriptions, by being a very wonderful mentor to me. His care, training and advice over the past several years have helped me foster my personal and professional skills, thus enabling me to become a stronger individual and an independent scientist. I am deeply indebted to him for making my stay in his laboratory a unique and unforgettable experience.

In addition to his guidance, the groundwork of this dissertation was supervised by an excellent team of very experienced members of my committee, consisting of Sidney Kushner, Michael Terns, Robert Ivarie and Richard Meagher. Over the years, they have motivated me and provided critical input, which further enhanced my research career and shaped my personality. I would specially like to thank Dr. Kushner, for being my inspiration and for being a 'parent' to me in the US. I am grateful to him for gently

molding my personality and for helping me discover myself. In addition, I would also like to thank him for offering us (Raj and me), invaluable emotional and moral support during very critical times. I will also always look back very fondly of my interactions with ‘Dear M&M’ as a wonderful experience, as I continue to move on with my life.

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I would not have made it this far, if not for the beautiful and neurotic moments spent with all the undergraduates, innumerable friends, classmates and colleagues, during my saga in this lovely and dynamic department.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

"Asked a human to a yeast, one day,
What is 'Cap' and what does it do, I pray?
Said, the yeast to the human that day,
It is essential for life, as much as I can say.
For Cap provides end protection,
From activities, like NHEJ and recombination;
And Cap also assists in length regulation,
By allowing access to telomerase, for telomere addition;
For Cap controls the state of beings, you see,
Along with DNA and proteins, it opens and closes the ends of me.
And Cap forms protective structures, quite precisely,
Popularly named 't-loops' for the whole world to see."

THE ENDS ARE SPECIAL: WHEN DID THEY ARISE?

The concept of evolution is that all life has been derived from a common ancestor. But one can ask, “What is the essence of life and how is it preserved?” The answers to these questions are not trivial. There are many rival theories on the account of how life originated and was preserved; yet all have a common thread running through them. Life can be based on DNA or RNA molecules, which are capable of encoding all the information needed to make a complete genome (41). This dissertation focuses on the challenges organisms undergo in maintaining a functional genome.

The rise of linear chromosomes and more complex life forms brought about new challenges in preserving the genome. Unlike their prokaryotic ancestors with mostly circular genomes, complex eukaryotes had to come up with ways for differentiating the natural linear ends from double-stranded DNA breaks (DSB) induced by damages, to prevent them from fusing to each other. With the advent of microscopic techniques, a great deal of information has been derived by observing the behavior of intact and broken DNA of *Drosophila* and Maize that were generated by X-ray damage (54, 62). It was observed that unlike DSBs, the naturally occurring ends of linear genomes were special, which Muller named the telomere because they provided a stable protective function (62).

The concept that linear ends of the genomes were ‘capped’ or protected from fusing to other ends was thus spawned. This ‘capping’ property was the first essential function ascribed to the telomeres. The other complex property later assigned to the telomere was to find a way to solve the problem caused by shortening of telomeres with every round of DNA replication. In 1985, the discovery of an RNA-dependent DNA polymerase activity in an enzyme called

telomerase, by Elizabeth Blackburn and Carol Grieder, provided the first set of clues for solving the problem of end maintenance (35).

We now know that telomeres are specialized DNA-protein caps, consisting of tandem DNA repeats present at the ends of linear eukaryotic chromosomes. In most eukaryotic cells, telomerase counterbalances natural telomere shortening by adding repeats to the ends, thus contributing to end maintenance. In humans, telomeres also have a tumor suppressor function and are intrinsically linked to cellular immortality and oncogenesis.

The focus of this literature review will be on telomere research conducted with yeast, in particular, *Kluyveromyces lactis* (*K. lactis*), the model organism chosen for this study. As an additional source of reference, examples of other systems will be cited.

CHROMOSOME END PROTECTION

In most eukaryotes, telomeric DNA is composed of repetitive sequences of G (guanine) and C (cytosine) rich strands. The telomere sequences can vary in length, and one specific to the particular organisms (see Table 1-1). One such example is the budding yeast *K. lactis*, which contains ~300 base pairs of identical blocks of 25 base pair sequences, repeated in a tandem array. Other organisms have telomeric repeats ranging from 5-26 bp (89). The *K. lactis* repeat is copied from a 30 nt template region of the telomerase RNA (56). Telomeric sequences in many or all organisms end in a small single-stranded 3' tail or overhang of varying length. The length of the overhangs is important because they serve as a platform for many proteins to bind, thus providing protection by masking the very tip of the chromosome.

Hence, one can say that function of chromosome end protection, involves proteins interacting with the telomere DNA, which in turn stabilizes the chromosome ends, to prevent

fusions by the non homologous end joining pathways and from homologous recombination repair activities (25). In addition to these two important components, the DNA protein complexes, also allow regulated access to telomere maintenance pathways.

A. Telomere DNA Overhang

The cell's DNA replication machinery results in the generation of a short 3' overhang at the telomere because of the removal of the last RNA primer from the terminal Okazaki fragment (9). Direct evidence for the presence of overhangs at the tips of the chromosomes first came from electron microscopic analysis of trypanosome telomeres coated with the *Escherichia coli* SSB protein (63). On the basis of the number of SSB proteins bound to the telomeric DNA, the length of the overhang was determined to be ~100 nucleotides in length (63). Although the length of the overhang in wild type cells of *K. lactis* is unknown, in *S. cerevisiae,* the variable single-stranded G-overhangs have been shown to form in a cell-cycle dependent manner, with approximately 30 nucleotides in S phase and 14 nucleotides in G1/G2 phases (see Table 1-1) (21, 101).

One of the major consequences of telomere uncapping is the deregulation of overhangs at the telomeric tip through the further resection of the telomeric C-rich strand. Research work in *S. cerevisiae* can be drawn to illustrate the function of a compromised cap. Very long single-stranded overhangs are found throughout the cell cycle in some mutants with a defective Cdc13 telomere binding protein (29, 51). This illustrates that long single-stranded overhangs extending from the telomere occur at a high level in mutants with uncapped telomeres. Supporting this idea is recent work in *K. lactis TER1* template mutants with long dysfunctional telomeres, which also display a substantial amount of single stranded telomeric DNA; consistent with a capping

malfunction (37, 99). All these examples emphasize the fact that the capping function is intrinsically dependent on the telomere DNA sequence.

Rare exceptions to canonical telomeric repeats are seen in certain plants and insect species. In *Drosophila* for instance, chromosomes ends are provided by certain retrotransposable elements (71). In other organisms like the fission yeast, *S. pombe*, which lack uniform telomere repeats, the telomere binding protein Taz1, and the heterochromatin protein, Swi6, can remain associated with chromosome ends even in the absence of telomere sequences (77). Such examples allude to the importance of heterochromatin factors contributing to the function of telomere end protection.

B. Protein complexes protecting the ends

How capping is achieved is not fully clear, but a number of proteins binding to the single or double-stranded regions of the telomeres are known to be involved in regulating this process (Fig. 1-1) (12, 26, 58, 81). It is believed that telomeres regulate the capping function by maintaining two configurations during the progression of the cell cycle (8). During DNA replication at S phase, the telomere adopts an open state allowing action of telomerase at the 3' termini. By allowing proper regulation of telomere replication, telomere proteins help provide a protective capping function to an organism.

In both budding yeasts *S. cerevisiae* and *K. lactis*, the double-stranded DNA binding protein Rap1 has been shown to interact with other telomeric proteins to mediate end protection and regulate telomere length. In *K. lactis*, mutations in the template region of the telomerase RNA (*TER1*) have been used to study the capping function at the telomere (99). Other results have indicated that telomere uncapping occurs in some *TER1* mutants that have been implied to

diminish Rap1 binding, while promoting chromosome circularization despite retaining some of the telomere sequences (58). This implies that removal of a protective protein at the very end of a telomere, rather than a complete loss of telomere sequences, is sufficient to bring about a loss of telomere capping.

Similarly, in *S. cerevisiae*, Rap1 is required for chromosome stability and prevention of fusion formation at the telomere (18, 70). Additionally, Rif1p and Rif2p (Rap1 interacting factors), along with the Sir protein complex, which is involved in silencing of the genes, interact with the C-terminal domain of Rap1 (70). Together this complex provides a sensitive regulator of telomerase to access the ends and add repeats. When telomere length exceeds a certain threshold, the binding of excess Sc.Rap1 molecules acts to prevent access by telomerase. Although both telomerase access and capping are regulated by Rap1, capping appears to require fewer telomere repeats and is therefore a separate function (24).

Additionally, mutants with defective proteins that are involved in other DNA repair processes are increasingly being shown to effect capping functions at the telomere. The paradoxical roles of key proteins like Ku80 and Mre-11, Rad50, Xrs2 (MRX complex) in the NHEJ pathway and telomere capping and repair are still unclear. Research work in *S. cerevisiae* has demonstrated that Ku does have a role in end protection at the telomeres. Disruption of the Ku subunits resulted in telomere shortening while at the same time the single-stranded overhangs became longer (5, 6, 10, 34, 91). Cells deleted for Ku are temperature-sensitive (2, 10) causing a RAD9-dependent checkpoint arrest due to increased amount of single-stranded DNA (91). In Ku mutant strains, the single-strand overhangs are present throughout the cell cycle, as compared with the cell- cycle regulated fluctuations in the overhangs found in wild-type cells (34). These results led to the conclusion that the Ku complex is involved as part of the cap structure at the

telomere. The Rad50/ Mre11/ Xrs2 (MRX) protein complex, has also been thought to play a role in end protection although much of the data remains to clarified (49).

Although a complete picture of the role of the individual NHEJ components for telomere end protection is still in its infancy, initial attempts to elucidate their function have been carried out in this dissertation and elsewhere in other organisms (70). A list of all the major players involved in the repair pathway is provided in Table 1-2.

Although a lot of the work on capping has focused on the double- stranded region of the telomere, the single-stranded part comprised of a short sequence of telomeric DNA, is also critically important, because it is acts as the main functional capping complex. There are a number of proteins that bind to the single-stranded overhang to protect the telomere. Extensive work in *S. cerevisiae*, has shown that Cdc13p, Stn1p and Ten1p appear to be important components of the telomere cap complex (24). Consistent with a capping function, a mutation in *CDC13* leads to elevated levels of telomere recombination and long 3' overhangs (22). Recruitment of Stn1p to the telomere can suppress a null mutant of *CDC13*, indicating that it is necessary for end protection (33, 72). Stn1p has also been shown both genetically and biochemically to interact with Ten1p *in vivo* (32). *ten1* mutant alleles, like *stn1* alleles, can lead to significant telomere elongation, suggesting that they function together in telomere capping and length regulation (Figure 1-1).

In order to gain an in depth understanding of the capping complex, the *STN1*and *CDC13* genes have been cloned and identified in *K. lactis* [(42), O.Sprusansky, C. Davis and M.J.McEachern , unpublished data]. A telomeric DNA phenotype consistent with a capping defect has been observed in the *stn1-M1* mutant in *K. lactis* (42). The role of the *K. lactis CDC13*

gene in end protection and whether *stn1-M1* perturbs the interaction with *CDC13*, awaits characterization.

C. End Structure

Given the overlap between proteins regulating DSB repair elsewhere in the genome in addition to playing a role in telomere capping, one can hypothesize that, organisms evolved ways to form compact higher order protective structures through looping or folding of the telomere tract. These structures could play a role in clearly defining the chromosome end *vs.* a double stranded DNA break (DSB), even if the same set of proteins bound both the DNA molecules. The complexity of the structure, depending mostly on the length of 3' overhang and the number of proteins bound to these overhangs, could in turn actively change the architecture of the resulting end. Organisms have adopted a number of ways to stabilize a linear chromosome. For the purpose of this dissertation, a few examples in yeast and humans, will be discussed in the following sections.

Early evidence in *S. cerevisiae* indicated the existence of fold-back structures mediated by Rap1 interaction with the Sir proteins (19). This fold-back structure is thought to be necessary to repress expression of the sub-telomeric genes, thus playing a role in establishing telomere silencing (TPE) in yeast.

However, in a variety of species, including humans, the telomeres have been postulated to form a protective structure, where the 3'end is thought to strand invade into the internal duplex region of the telomere to form a telomere loop (t-loop), (13, 36, 63, 67, 94, 95). It is unclear if the 3' overhangs in budding yeasts invades the duplex telomere tract to form protective t-loops. However, given the divergence in telomere sequences and telomeric proteins from yeast

to man, it is reasonable to believe that budding yeast telomeres may adopt a different protective structure.

CHROMOSOME END MAINTENANCE

A. Telomerase

Yeasts use telomerase, as a major component in telomere length regulation. This ribo-nucleoprotein enzyme, mainly is composed of an RNA template and a protein subunit (59). The RNA component is called *Tlc1* in *S. cerevisiae* (23) and *Ter1* in *K. lactis* (55) and TR in all other organisms (15). The RNA template sequence, which is A/C-rich in most species, leads to the synthesis of T/G-rich telomeric DNA. The protein catalytic subunit called Est2 in *S. cerevisiae* and *K. lactis* and TERT in all other organisms, uses the template to add telomeric repeats to the very ends of the chromosomes (59, 98).

B. Other protein complexes

In addition to the core components of telomerase, other genes like Est1 and 3, Yku 70/80, Cdc13, Stn1 and Ten1, also play overlapping roles in telomere end protection and length regulation (23, 24, 59, 75, 88). Most of our knowledge, on telomerase mediated telomere maintenance comes from work carried out in *S. cerevisiae*, and will be discussed in the subsequent section.

In *S. cerevisiae* where length regulation has been extensively studied, Cdc13, a single-stranded telomere end binding protein has been postulated to play dual roles in telomere length regulation by telomerase. The first role is positive regulation of the telomerase in association with Est1, Est13 and the Ku complex. Cdc13 loads on to single-stranded telomeric 3'overhang

and serves as a platform for telomerase recruitment by binding to Est1, in a cell cycle dependent manner (14, 23, 30). Ku also plays a positive role, independently of Cdc13, in regulating telomerase, by interacting with a stem loop structure of Tlc1 and by binding to the double-stranded region of the telomeric DNA (82).

The other function of Cdc13 is to prevent telomerase from adding repeats at the chromosome ends. It has been proposed that this negative regulation is achieved through a protein complex comprising of Stn1 and Ten1. They associate with Cdc13 inhibiting telomerase access and thus allowing the DNA polymerase alpha to complete the lagging strand synthesis and fill in the bulk of the 3' overhang (14, 24, 31, 33, 38). Additional proteins such as Rap1, a double-stranded telomeric DNA binding protein in both yeasts, are also involved in negative regulation of telomerase by inhibiting telomerase mediated telomeric extension (48). Research has shown that mutations in the telomere that disrupt Rap1 binding can lead to severe telomere elongation in *S. cerevisiae* (74) and in *K. lactis* (44, 45). Telomere length is regulated by a negative feedback mechanism where the number of molecules of Rap1p bound to the telomere has an inverse effect on the control of telomere length.

C. Non Homologous End Joining

In recent years, it has been shown that there are other ways to maintain chromosomes in the absence of telomerase. One such example is circularization of chromosomes by the non-homologous end-joining pathway (NHEJ). Fusions or circularization between telomeres occur as a consequence of a repair mechanism when the protective capping function is lost or compromised.

Well-studied examples of such events in cells lacking telomerase have been observed in the fission yeast *S. pombe*. Most cells lacking the catalytic subunit of telomerase die, but rare emerging survivors appear as result of circularization of the three chromosomes by covalent fusions between them (64). Other notable examples of yeast mutants exhibiting telomere fusions have come from studies in budding yeasts, *K. lactis* and *S. cerevisiae*. Circularization of all six chromosomes appears to take place in *K. lactis* strains with mutations in the template region of the telomerase RNA, which are predicated to abolish or greatly decrease Rap1 binding (58). Recent work by Pardo and Marcand in 2005, demonstrated that Rap1 was required to avoid fusion formation in *S. cerevisiae* (70). It is not known whether these fusions led to circularization of chromosomes.

Telomere-telomere fusions in both yeast appear to arise by an NHEJ-dependent mechanism since these fusions require Lig4, a NHEJ-specific ligase, in both yeasts (25, 50, 61). There are three main protein complexes known to mediate NHEJ processes; the Ku heterodimer, the MRX complex, and DNA ligase IV (reviewed in (97). Lif1, a minor player is the yeast functional homologue of mammalian Xrcc4 is required for the stability and full activity of Lig4 (92). The *NEJ1* gene, encodes a protein that interacts with Lif1 and is essential for NHEJ and is known to be required for fusions between telomeres (28).

D. Recombination

Prior to telomerase, recombination may have been an alternate form of telomere maintenance and research work in *K. lactis* and *S. cerevisiae* has been very important in understanding it. The most prominent alternate pathway is recombinational telomere elongation (RTE), which uses recombination to replicate and lengthen the ends of chromosomes. Most of

the mechanisms of RTE are still shrouded in mystery. However, the best description has come from the work done in *S. cerevisiae* and *K. lactis* by observing and characterizing the properties of post-senescent survivors, which arise upon the deletion of telomerase (55, 80). Cells lacking telomerase, after an initial phase of gradual telomere shortening and cell death, produce healthier colonies that have restored telomeric repeat arrays to varying degrees through homologous recombination. There are two types of RTE pathways observed in *S. cerevisiae* due to its unique telomeric structure comprising of Y' subtelomeric elements interspersed with telomeric $TG_{(1-3)}$ repeats (16, 90) (Figure 1-2). Type I involves amplification of internal Y' segments of the telomere while the telomeric ends are maintained at short lengths (16). However, Type II involves amplification of only the $TG_{(1-3)}$ telomeric repeats (16, 90). These two pathways have been distinguished by their differing requirement for a number of recombination genes namely *RAD52, RAD51*, *RAD54*, and *RAD57* (for Type I) and *RAD52, RAD59 RAD50, MRE11, XRS2, SGS1* (for Type II) (16, 69, 90).

In *K. lactis,* just Type II survivors normally occur in telomerase deletion mutants. Unlike *S. cerevisiae*, *K. lactis* lacks Y' internal segments, and only contains homogenous telomere sequences. The genetic requirements for the RTE pathway in *K. lactis* are just beginning to be unraveled, and they appear to be different from those of *S. cerevisiae*. The gene *RAD59*, which has been shown to be absolutely essential for type II elongation in *S. cerevisiae,* is not completely required for survivor formation in *K. lactis* (S.Iyer and M.McEachern, unpublished data). Work is currently underway to identify, clone and characterize other genes involved in RTE in *K. lactis*.

A second type of RTE has been recently shown to occur in *K. lactis* (Figure 1-2). We have defined it as type IIR (type II runaway) and is the major focus of this dissertation (42). The

telomere elongation by this atypical recombination pathway, is of an extreme nature and is different from the modest lengthening observed in *K. lactis* cells deleted for telomerase (42). Other examples of RTE resembling type IIR has been observed in certain mutants that contain mutant telomere repeats [(96) and L. Harris, Topcu. Z and M. McEachern manuscript in preparation]. It will be very interesting to determine the mechanism of type II R RTE in *K. lactis*.

At least a few other organisms and some plants maintain their telomeres by recombination. The midge *Chironomus* contains complex repeats arranged in tandem and lacks simple repeated sequences at its chromosomal termini (17). In *A. gambiae* (mosquito) (7) and *A. cepa* (onion) (73), telomere maintenance seems to be promoted by recombination. Recently, mitochondria with linear chromosomes have been found with varying copies of direct repeats that function as telomeres in *Candida parapsilopsis* and certain other Candida species (68). A recombination-dependent mechanism has been proposed to play a role in the maintenance of their telomeres (93).

MODELS TO EXPLAIN RTE

A: Break Induced Replication: BIR only

Homologous recombination in yeast and other organisms appears to be promoted by DNA double-strand repair (87). The DSB repair process includes, processing of DNA strands bi-directionally by a single-strand exonuclease to produce 3' ended tails. The free 3' tails invade an intact homologous donor sequence to form a displacement loop (D-loop) thus priming DNA replication. The next step is a DNA heteroduplex migration, creating two Holliday junctions. Resolution of these junctions by filling in the gaps can lead to the production of two intact crossover or non- crossover products (87). Alternatively, displacement of the invaded ends

followed by their annealing can bring about gene conversion with no crossover. However, recent studies in *S. cerevisiae*, using non telomeric broken DNA ends have alluded to a related repair process called break-induced replication (BIR), which can extend a single broken end by copying an entire chromosome arm up to several hundred kilobases in length in a unidirectional manner (Figure 1-3A) (46, 52, 60, 78). Although BIR has been suggested to be involved in recombinational telomere lengthening, the process and mechanism of BIR at and around the telomeres is still in its infancy and subject to speculation.

B. The Roll and Spread model

Important insights into the mechanism of RTE came from experimental work conducted in *K. lactis*. To explain telomere lengthening in *K. lactis*, a two-step mechanism was postulated that involved rolling circle replication followed by intermolecular recombination or BIR events (66). The first step was creation of a circular template by an intra molecular recombination event that was subsequently utilized to lengthen a single telomere. The second step was spreading the elongated telomere sequence to other telomeres by intermolecular BIR events (See Figure 1-3B). Experiments were carried out to test the model by introducing a 1.6 Kb DNA circle containing a telomeric repeat sequence and a *URA3* marker gene. Such circles led to the formation of telomeric tandem arrays of telomere and URA3 sequence. The process of array formation was mostly dependent on the *RAD52* gene. The elongated sequence derived from the DNA circle was spread to other telomeres very efficiently in telomerase deletion cells (65, 96). Additional data has shown that *RAD52*- dependent telomere circles as small as ~100 bp can form in a *TER1* mutant with long dysfunctional telomeres elongated by telomerase and also in a second class of mutant exhibiting type IIR RTE, the main focus of chapter 3 (37). It will be very interesting to

determine if these circles contribute to or are the consequence of, telomere capping defects in both the mutants and whether they contribute to type IIR in *K. lactis*.

SIGNIFICANCE OF THIS WORK FOR HUMAN HEALTH: CANCER & AGING

The earliest report linking telomere biology to carcinogenesis came from the observation that cancer cells have shorter telomeres than their normal counterparts (20). In normal somatic cells, mechanisms for maintaining telomere length are absent due to the absence or low levels of telomerase activity (40). Therefore, with each successive round of cell division, telomeres progressively shorten, losing up to 200 base pairs of terminal DNA per cell division from the tips of their chromosomes (40). Ultimately, continuing number of cell divisions further shortens the length and uncaps the telomeres, triggering a non-dividing state known as replicative senescence. Therefore, a central question one might be tempted to ask is whether short uncapped telomeres ultimately lead to formation of tumors cells or if they transiently suppress tumors by triggering replicative senescence.

An attractive hypothesis is that replicative senescence can be thought of as a temporary cancer suppressor mechanism, although some cells bypass this stage with continued telomere shortening (39). This leads to increased genomic instability, including cell death, chromosome end fusions and activation of telomerase, leading to the development of cancer (43). Although most cancer cells are immortalized by telomerase, a subset of the cancer cells are immortalized by an alternate pathway (ALT), which is characterized by long telomeres lengthened by recombination (76). Currently, no mutations have been identified that directly trigger the ALT pathway in humans. Thus, the exact molecular mechanism (s) triggering ALT still remains to be elucidated.

To better understand the role of telomeres and telomerase in cancer development, experiments were conducted in mice to provide further clues regarding increased cancer risk with advancing human age (4, 11, 53, 79). Recent evidence has linked shorter telomeres to the aging of human cells (3). Shortened telomeres have been identified in tissues associated with a variety of diseases including atherosclerosis, hepatitis and blood disorders (1, 39, 47, 53). Accelerated telomere shortening, together with a decreased replicative life span has been linked to several aging syndromes including Werner syndrome (WS), Bloom syndrome (BS) and Down syndromes (11, 85, 100). Such data has led to a growing belief that replicative senescence arising from telomere shortening could also be an important contributor to aging in human tissues (3). Other studies have shown that expression of telomerase could increase cell division capacity, thus preventing replicative senescence (47, 53, 83, 84). It is reasonable to think that stimulating telomere elongation will relieve or prevent some symptoms of aging.

As such, research work carried in this dissertation on understanding RTE pathways in *K. lactis* could be significant in providing clues towards understanding the molecular mechanisms triggering age related diseases and ALT in human cells. Additionally, the capping function of telomeres can be thought to play a significant role in protecting against the development of many types of cancers, while uncapping of telomeres and activating the telomerase or ALT pathways could play a role in maintaining the immortal state of cancer cells. It is not surprising to note that beside telomerase and recombination proteins, telomere capping proteins could be considered as attractive targets for cancer therapy.

OBJECTIVES OF THIS STUDY

The overall objective of this study was to develop a better understanding of how functional caps at the ends of chromosomes suppress activities like recombinational repair and non-homologous end-joining. Our working hypothesis was that mutating the genes integral to the capping-complex would trigger elevated HR and NHEJ activity at the telomeres. To test this hypothesis we conducted studies to address the following specific objectives.

Objective1: To isolate and characterize a mutation in the gene of the capping-complex, triggering type IIR RTE in *K. lactis*.

The effects of this mutation in yeast, is similar to what has been observed in ALT cells in human cancers, therefore providing an attractive model to understand the genes regulating ALT.

Objective 2: To provide evidence of recombinational products in a mutant undergoing type IIR RTE.

We have provided preliminary evidence of DNA structures resembling rolling circle replication intermediates in a mutant that exclusively elongates its telomeres by recombination. The presence of large telomeric circles could allude to the possibility of these circles being a major contributor for driving type IIR RTE in several different organisms.

Objective 3: To characterize the effects of NHEJ mutants on telomere metabolism in *K. lactis*.

Results from this work demonstrate the involvement of components of NHEJ pathway in providing telomeric end protection.

Overall, this study was expected to establish a new focus on end protection independent of the initial length of the telomere. Loss of end protection triggers not only an unusual RTE pathway in *K. lactis*, but more plausibly provides clues of how ALT is activated in humans; an area of intense investigation in the field of telomere biology. Furthermore, positive results were

expected to provide important mechanistic clues to the RTE and NHEJ pathways that get activated at telomeres with a compromised cap.

REFERENCES

1. **Baerlocher, G. M., A. Roth, and P. M. Lansdorp.** 2003. Telomeres in hematopoietic stem cells. Ann N Y Acad Sci **996:**44-8.

2. **Barnes, G., and D. Rio.** 1997. DNA double-strand-break sensitivity, DNA replication, and cell cycle arrest phenotypes of Ku-deficient Saccharomyces cerevisiae. Proc Natl Acad Sci U S A **94:**867-72.

3. **Baur, J. A., J. W. Shay, and W. E. Wright.** 2004. Spontaneous reactivation of a silent telomeric transgene in a human cell line. Chromosoma.

4. **Ben-Porath, I., and R. A. Weinberg.** 2004. When cells get stressed: an integrative view of cellular senescence. J Clin Invest **113:**8-13.

5. **Bertuch, A. A., and V. Lundblad.** 2003. The Ku heterodimer performs separable activities at double-strand breaks and chromosome termini. Mol Cell Biol **23:**8202-15.

6. **Bertuch, A. A., and V. Lundblad.** 2003. Which end: dissecting Ku's function at telomeres and double-strand breaks. Genes Dev **17:**2347-50.

7. **Biessmann, H., J. Donath, and M. F. Walter.** 1996. Molecular characterization of the Anopheles gambiae 2L telomeric region via an integrated transgene. Insect Mol Biol **5:**11-20.

8. **Blackburn, E. H.** 2000. Telomere states and cell fates. Nature **408:**53-6.

9. **Blackburn, E. H.** 1994. Telomeres: no end in sight. Cell **77:**621-3.

10. **Boulton, S. J., and S. P. Jackson.** 1996. Identification of a Saccharomyces cerevisiae Ku80 homologue: roles in DNA double strand break rejoining and in telomeric maintenance. Nucleic Acids Res **24:**4639-48.

11. **Burkle, A.** 2002. In memoriam Bernard Strehler--genomic instability in ageing: a persistent challenge. Mech Ageing Dev **123:**899-906.

12. **Cervantes, R. B., and V. Lundblad.** 2002. Mechanisms of chromosome-end protection. Curr Opin Cell Biol **14:**351-6.

13. **Cesare, A. J., N. Quinney, S. Willcox, D. Subramanian, and J. D. Griffith.** 2003. Telomere looping in P. sativum (common garden pea). Plant J **36:**271-9.

14. **Chandra, A., T. R. Hughes, C. I. Nugent, and V. Lundblad.** 2001. Cdc13 both positively and negatively regulates telomere replication. Genes Dev **15:**404-14.

15. **Chen, J. L., and C. W. Greider.** 2004. An emerging consensus for telomerase RNA structure. Proc Natl Acad Sci U S A **101:**14683-4.

16. **Chen, Q., A. Ijpma, and C. W. Greider.** 2001. Two survivor pathways that allow growth in the absence of telomerase are generated by distinct telomere recombination events. Mol Cell Biol **21:**1819-27.

17. **Cohn, M., and J. E. Edstrom.** 1992. Chromosome ends in Chironomus pallidivittatus contain different subfamilies of telomere-associated repeats. Chromosoma **101:**634-40.

18. **Conrad, M. N., J. H. Wright, A. J. Wolf, and V. A. Zakian.** 1990. RAP1 protein interacts with yeast telomeres in vivo: overproduction alters telomere structure and decreases chromosome stability. Cell **63:**739-50.

19. **de Bruin, D., S. M. Kantrow, R. A. Liberatore, and V. A. Zakian.** 2000. Telomere folding is required for the stable maintenance of telomere position effects in yeast. Mol Cell Biol **20:**7991-8000.

20. **de Lange, T., L. Shiue, R. M. Myers, D. R. Cox, S. L. Naylor, A. M. Killery, and H. E. Varmus.** 1990. Structure and variability of human chromosome ends. Mol Cell Biol **10:**518-27.

21. **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:**13902-7.

22. **DuBois, M. L., Z. W. Haimberger, M. W. McIntosh, and D. E. Gottschling.** 2002. A quantitative assay for telomere protection in Saccharomyces cerevisiae. Genetics **161:**995-1013.

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

24. **Evans, S. K., and V. Lundblad.** 2000. Positive and negative regulation of telomerase access to the telomere. J Cell Sci **113 Pt 19:**3357-64.

25. **Ferreira, M. G., and J. P. Cooper.** 2004. Two modes of DNA double-strand break repair are reciprocally regulated through the fission yeast cell cycle. Genes Dev **18:**2249-54.

26. **Ferreira, M. G., K. M. Miller, and J. P. Cooper.** 2004. Indecent exposure: when telomeres become uncapped. Mol Cell **13:**7-18.

27. **Fisher, T. S., and V. A. Zakian.** 2005. Ku: a multifunctional protein involved in telomere maintenance. DNA Repair (Amst) **4:**1215-26.

28. **Frank-Vaillant, M., and S. Marcand.** 2001. NHEJ regulation by mating type is exercised through a novel protein, Lif2p, essential to the ligase IV pathway. Genes Dev **15:**3005-12.

29. **Garvik, B., M. Carson, and L. Hartwell.** 1995. Single-stranded DNA arising at telomeres in cdc13 mutants may constitute a specific signal for the RAD9 checkpoint. Mol Cell Biol **15:**6128-38.

30. **Grandin, N., C. Damon, and M. Charbonneau.** 2000. Cdc13 cooperates with the yeast Ku proteins and Stn1 to regulate telomerase recruitment. Mol Cell Biol **20:**8397-408.

31. **Grandin, N., C. Damon, and M. Charbonneau.** 2001. Cdc13 prevents telomere uncapping and Rad50-dependent homologous recombination. Embo J **20:**6127-39.

32. **Grandin, N., C. Damon, and M. Charbonneau.** 2001. Ten1 functions in telomere end protection and length regulation in association with Stn1 and Cdc13. Embo J **20:**1173-83.

33. **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.

34. **Gravel, S., M. Larrivee, P. Labrecque, and R. J. Wellinger.** 1998. Yeast Ku as a regulator of chromosomal DNA end structure. Science **280:**741-4.

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

36. **Griffith, J. D., L. Comeau, S. Rosenfield, R. M. Stansel, A. Bianchi, H. Moss, and T. de Lange.** 1999. Mammalian telomeres end in a large duplex loop. Cell **97:**503-14.

37. **Groff-Vindman, C., A. J. Cesare, S. Natarajan, J. D. Griffith, and M. J. McEachern.** 2005. Recombination at long mutant telomeres produces tiny single- and double-stranded telomeric circles. Mol Cell Biol **25:**4406-12.

38. **Grossi, S., A. Puglisi, P. V. Dmitriev, M. Lopes, and D. Shore.** 2004. Pol12, the B subunit of DNA polymerase alpha, functions in both telomere capping and length regulation. Genes Dev **18:**992-1006.

39. **Harley, C. B.** 1997. Human ageing and telomeres. Ciba Found Symp **211:**129-39; discussion 139-44.

40. **Harley, C. B., A. B. Futcher, and C. W. Greider.** 1990. Telomeres shorten during ageing of human fibroblasts. Nature **345:**458-60.

41. **Hayes, J. M.** 1996. The earliest memories of life on Earth. Nature **384:**21-2.

42. **Iyer, S., A. D. Chadha, and M. J. McEachern.** 2005. A mutation in the STN1 gene triggers an alternative lengthening of telomere-like runaway recombinational telomere elongation and rapid deletion in yeast. Mol Cell Biol **25:**8064-73.

43. **Kim, N. W., M. A. Piatyszek, K. R. Prowse, C. B. Harley, M. D. West, P. L. Ho, G. M. Coviello, W. E. Wright, S. L. Weinrich, and J. W. Shay.** 1994. Specific association of human telomerase activity with immortal cells and cancer. Science **266:**2011-5.

44. **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.

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

46. **Kuzminov, A.** 1995. Instability of inhibited replication forks in E. coli. Bioessays **17:**733-41.

47. **Lansdorp, P. M.** 2000. Repair of telomeric DNA prior to replicative senescence. Mech Ageing Dev **118:**23-34.

48. **Larson, G. P., D. Castanotto, J. J. Rossi, and M. P. Malafa.** 1994. Isolation and functional analysis of a Kluyveromyces lactis RAP1 homologue. Gene **150:**35-41.

49. **Lewis, L. K., F. Storici, S. Van Komen, S. Calero, P. Sung, and M. A. Resnick.** 2004. Role of the nuclease activity of Saccharomyces cerevisiae Mre11 in repair of DNA double-strand breaks in mitotic cells. Genetics **166:**1701-13.

50. **Liti, G., and E. J. Louis.** 2003. NEJ1 prevents NHEJ-dependent telomere fusions in yeast without telomerase. Mol Cell **11:**1373-8.

51. **Lydall, D., and T. Weinert.** 1995. Yeast checkpoint genes in DNA damage processing: implications for repair and arrest. Science **270:**1488-91.

52. **Malkova, A., E. L. Ivanov, and J. E. Haber.** 1996. Double-strand break repair in the absence of RAD51 in yeast: a possible role for break-induced DNA replication. Proc Natl Acad Sci U S A **93:**7131-6.

53. **Mariani, E., A. Meneghetti, I. Formentini, S. Neri, L. Cattini, G. Ravaglia, P. Forti, and A. Facchini.** 2003. Different rates of telomere shortening and telomerase activity reduction in CD8 T and CD16 NK lymphocytes with ageing. Exp Gerontol **38:**653-9.

54. **McClintock, B.** 1939. The behaviour of successive nuclear divisions of a chromosome broken at meiosis. Genetics **25:**405-416.

55. **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.

56. **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.

57. **McEachern, M. J., and J. E. Haber.** 2006. Break-Induced Replication and Recombinational Telomere Elongation in Yeast. Annu Rev Biochem.

58. **McEachern, M. J., S. Iyer, T. B. Fulton, and E. H. Blackburn.** 2000. Telomere fusions caused by mutating the terminal region of telomeric DNA. Proc Natl Acad Sci U S A **97:**11409-14.

59. **McEachern, M. J., A. Krauskopf, and E. H. Blackburn.** 2000. Telomeres and their control. Annu Rev Genet **34:**331-358.

60. **Michel, B.** 2000. Replication fork arrest and DNA recombination. Trends Biochem Sci **25:**173-8.

61. **Mieczkowski, P. A., J. O. Mieczkowska, M. Dominska, and T. D. Petes.** 2003. Genetic regulation of telomere-telomere fusions in the yeast Saccharomyces cerevisae. Proc Natl Acad Sci U S A **100:**10854-9.

62. **Muller, H. J.** 1938. The remaking of chromosomes. The Collecting Net **8:**182-195.

63. **Murti, K. G., and D. M. Prescott.** 1999. Telomeres of polytene chromosomes in a ciliated protozoan terminate in duplex DNA loops. Proc Natl Acad Sci U S A **96:**14436-9.

64. **Nakamura, T. M., J. P. Cooper, and T. R. Cech.** 1998. Two modes of survival of fission yeast without telomerase. Science **282:**493-6.

65. **Natarajan, S., C. Groff-Vindman, and M. J. McEachern.** 2003. Factors influencing the recombinational expansion and spread of telomeric tandem arrays in Kluyveromyces lactis. Eukaryot Cell **2:**1115-27.

66. **Natarajan, S., and M. J. McEachern.** 2002. Recombinational telomere elongation promoted by DNA circles. Mol Cell Biol **22:**4512-21.

67. **Nikitina, T., and C. L. Woodcock.** 2004. Closed chromatin loops at the ends of chromosomes. J Cell Biol **166:**161-5.

68. **Nosek, J., N. Dinouel, L. Kovac, and H. Fukuhara.** 1995. Linear mitochondrial DNAs from yeasts: telomeres with large tandem repetitions. Mol Gen Genet **247:**61-72.

69. **Paques, F., and J. E. Haber.** 1999. Multiple pathways of recombination induced by double-strand breaks in Saccharomyces cerevisiae. Microbiol Mol Biol Rev **63:**349-404.

70. **Pardo, B., and S. Marcand.** 2005. Rap1 prevents telomere fusions by nonhomologous end joining. Embo J **24:**3117-27.

71. **Pardue, M. L., O. N. Danilevskaya, K. Lowenhaupt, F. Slot, and K. L. Traverse.** 1996. Drosophila telomeres: new views on chromosome evolution. Trends Genet **12:**48-52.

72. **Pennock, E., K. Buckley, and V. Lundblad.** 2001. Cdc13 delivers separate complexes to the telomere for end protection and replication. Cell **104:**387-96.

73. **Pich, U., and I. Schubert.** 1998. Terminal heterochromatin and alternative telomeric sequences in Allium cepa. Chromosome Res **6:**315-21.

74. **Prescott, J. C., and E. H. Blackburn.** 2000. Telomerase RNA template mutations reveal sequence-specific requirements for the activation and repression of telomerase action at telomeres. Mol Cell Biol **20:**2941-8.

75. **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:**1777-88.

76. **Reddel, R. R.** 2003. Alternative lengthening of telomeres, telomerase, and cancer. Cancer Lett **194:**155-62.

77. **Sadaie, M., T. Naito, and F. Ishikawa.** 2003. Stable inheritance of telomere chromatin structure and function in the absence of telomeric repeats. Genes Dev **17:**2271-82.

78. **Seigneur, M., V. Bidnenko, S. D. Ehrlich, and B. Michel.** 1998. RuvAB acts at arrested replication forks. Cell **95:**419-30.

79. **Serrano, M., H. Lee, L. Chin, C. Cordon-Cardo, D. Beach, and R. A. DePinho.** 1996. Role of the INK4a locus in tumor suppression and cell mortality. Cell **85:**27-37.

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

81. **Smogorzewska, A., and T. de Lange.** 2004. Regulation of telomerase by telomeric proteins. Annu Rev Biochem **73:**177-208.

82. **Stellwagen, A. E., Z. W. Haimberger, J. R. Veatch, and D. E. Gottschling.** 2003. Ku interacts with telomerase RNA to promote telomere addition at native and broken chromosome ends. Genes Dev **17:**2384-95.

83. **Stewart, S. A., I. Ben-Porath, V. J. Carey, B. F. O'Connor, W. C. Hahn, and R. A. Weinberg.** 2003. Erosion of the telomeric single-strand overhang at replicative senescence. Nat Genet **33:**492-6.

84. **Stewart, S. A., and R. A. Weinberg.** 2002. Senescence: does it all happen at the ends? Oncogene **21:**627-30.

85. **Stewart, S. A., and R. A. Weinberg.** 2000. Telomerase and human tumorigenesis. Semin Cancer Biol **10:**399-406.

86. **Symington, L. S.** 2002. Role of RAD52 epistasis group genes in homologous recombination and double-strand break repair. Microbiol Mol Biol Rev **66:**630-70, table of contents.

87. **Szostak, J. W.** 1983. Replication and resolution of telomeres in yeast. Cold Spring Harb Symp Quant Biol **47 Pt 2:**1187-94.

88. **Taggart, A. K., S. C. Teng, and V. A. Zakian.** 2002. Est1p as a cell cycle-regulated activator of telomere-bound telomerase. Science **297:**1023-6.

89. **Teixeira, M. T., and E. Gilson.** 2005. Telomere maintenance, function and evolution: the yeast paradigm. Chromosome Res **13:**535-48.

90. **Teng, S. C., and V. A. Zakian.** 1999. Telomere-telomere recombination is an efficient bypass pathway for telomere maintenance in Saccharomyces cerevisiae. Mol Cell Biol **19:**8083-93.

91. **Teo, S. H., and S. P. Jackson.** 1997. Identification of Saccharomyces cerevisiae DNA ligase IV: involvement in DNA double-strand break repair. Embo J **16:**4788-95.

92. **Teo, S. H., and S. P. Jackson.** 2000. Lif1p targets the DNA ligase Lig4p to sites of DNA double-strand breaks. Curr Biol **10:**165-8.

93. **Tomaska, L., M. J. McEachern, and J. Nosek.** 2004. Alternatives to telomerase: keeping linear chromosomes via telomeric circles. FEBS Lett **567:**142-6.

94. **Tomaska, L., J. Nosek, A. M. Makhov, A. Pastorakova, and J. D. Griffith.** 2000. Extragenomic double-stranded DNA circles in yeast with linear mitochondrial genomes: potential involvement in telomere maintenance. Nucleic Acids Res **28:**4479-87.

95. **Tomaska, L., S. Willcox, J. Slezakova, J. Nosek, and J. D. Griffith.** 2004. Taz1 binding to a fission yeast model telomere: formation of telomeric loops and higher order structures. J Biol Chem **279:**50764-72.

96. **Topcu, Z., K. Nickles, C. Davis, and M. J. McEachern.** 2005. Abrupt disruption of capping and a single source for recombinationally elongated telomeres in Kluyveromyces lactis. Proc Natl Acad Sci U S A **102:**3348-53.

97. **Tsukamoto, Y., A. K. Taggart, and V. A. Zakian.** 2001. The role of the Mre11-Rad50-Xrs2 complex in telomerase- mediated lengthening of Saccharomyces cerevisiae telomeres. Curr Biol **11:**1328-35.

98. **Tzfati, Y., Z. Knight, J. Roy, and E. H. Blackburn.** 2003. A novel pseudoknot element is essential for the action of a yeast telomerase. Genes Dev **17:**1779-88.

99. **Underwood, D. H., C. Carroll, and M. J. McEachern.** 2004. Genetic dissection of the Kluyveromyces lactis telomere and evidence for telomere capping defects in TER1 mutants with long telomeres. Eukaryot Cell **3:**369-84.

100. **Von Zglinicki, T.** 2003. Replicative senescence and the art of counting. Exp Gerontol **38:**1259-64.

101. **Wellinger, R. J., A. J. Wolf, and V. A. Zakian.** 1993. Saccharomyces telomeres acquire single-strand TG1-3 tails late in S phase. Cell **72:**51-60.

TABLES

Table 1-1. Examples of telomere sequences and overhangs in Eukaryotes

Organism	Telomere Sequence	Telomere Length	Overhang Length (nt)
Human	TTAGGG	4-14 kb	100-200
Mus musculus	TTAGGG	20-150 kb	?
C. elegans	TTAGGC	4-9 kb	?
Bombyx	TTAGG	6-8 kb	?
Arabidopsis	TTTAGGG	2-5 kb	20-30
S. pombe	$TTAC(A)G_{2-8}$	200-300 bp	?
S. cerevisiae	TG_{1-3}	200-500 bp	~ 30 in S phase ~ 14 in G1/G2
K. lactis	TTGATTAGGTATGTGGTGTTCGGA	300-600 bp	?
Candida albicans	ACGGATGTCTAACTTCTTGGTGT	300-600 bp	?
Oxytricha	TTTTGGGG	20 bp	16
Tetrahymena	TTGGGG	300 bp	16
Trypanosoma brucei	TTAGGG	10-20	?

Data adapted from (89)

Table 1-2 Summary of key proteins involved in HR and NHEJ in *S. cerevisiae* and humans

Pathway	Yeast	Human	Function
Recombinational Repair- HR	Sgs1	WRN	DNA helicase- DNA unwinding
	Rad 51	Rad 51 A,B,C,D	RecA homolog-Strand invasion
	Rad 52	Rad 52	All homologous recombination
	Rad 54	Rad 54 L,B	Dissociates Rad 51 bound DNA
	Rad 55	-	Helps in Rad51mediated repair
	Rad 57	-	Helps in Rad51mediated repair
	Rad 59	-	Functional overlap with Rad52
HR and NHEJ	Rad 50	Rad 50	MRX complex- SMC like protein
	Mre 11	Mre 11	MRX complex- Nuclease activity
	Xrs2	NBS 1	MRN (X) complex
Non-homologous end joining NHEJ	YKu 80	hKu 80	DNA binding: also allows MRE11 to act in both HR and NHEJ activities
	YKu 70	hKu 70	
	-	DNA PKs	DNA dependent proteion kinase: recognises DSB
	Dnl 4/Lig 4	hLig 4	Special ligase associates with Ku
	Lif 1	Xrcc1	Accessory component of Lig4
	Nej1	-	Accessory component of Lif1

Most of the homologues have been identified in *K. lactis* and await further characterization. Reviewed in (27, 57, 69, 86).

FIGURES

Figure 1-1.

Schematic of a capped yeast telomere.

The double-stranded and the single-stranded components of the telomere are bound by many proteins. The single-stranded region of the telomere is bound by Cdc13. Est1, Stn1, Ten1 and components of telomerase interact with this part of the telomere. Stn1 is a negative regulator of telomerase. When it is bound to Cdc13 along with Ten1, it forms the functional cap at the very end of the chromosome. The double-stranded region of the telomere is bound by Rap1, which interacts with Sir protein complexes and Rif1 and Rif2. The junction of the double and single-stranded is bound by the Ku complex. Other proteins complexes like Mre11, Rad50 and Xrs2 are also involved in end protection and telomerase recruitment.

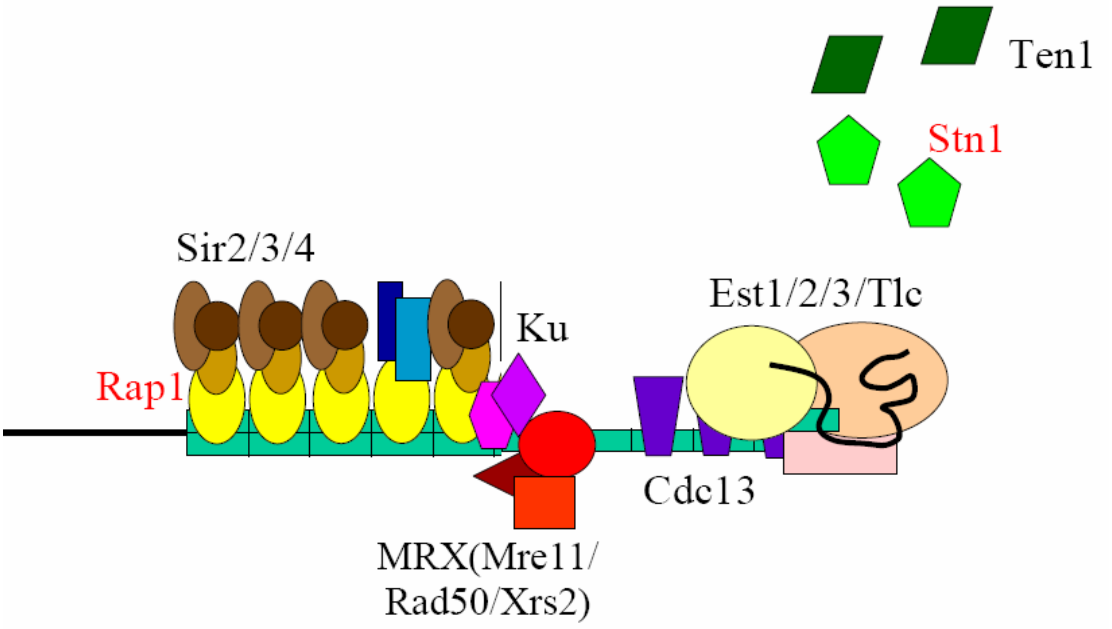
Ten1
Stn1
Sir2/3/4
Ku
Est1/2/3/Tlc
Rap1
Cdc13
MRX(Mre11/
Rad50/Xrs2)

Figure 1-1

Figure 1-2.

Recombinational telomere elongation pathways in yeast

Gradual telomere shortening in the absence of telomerase leads to the activation of Type I and Type II RTE pathways. Mutation of a capping protein leads to activation of Type IIR RTE pathway in the presence of telomerase.

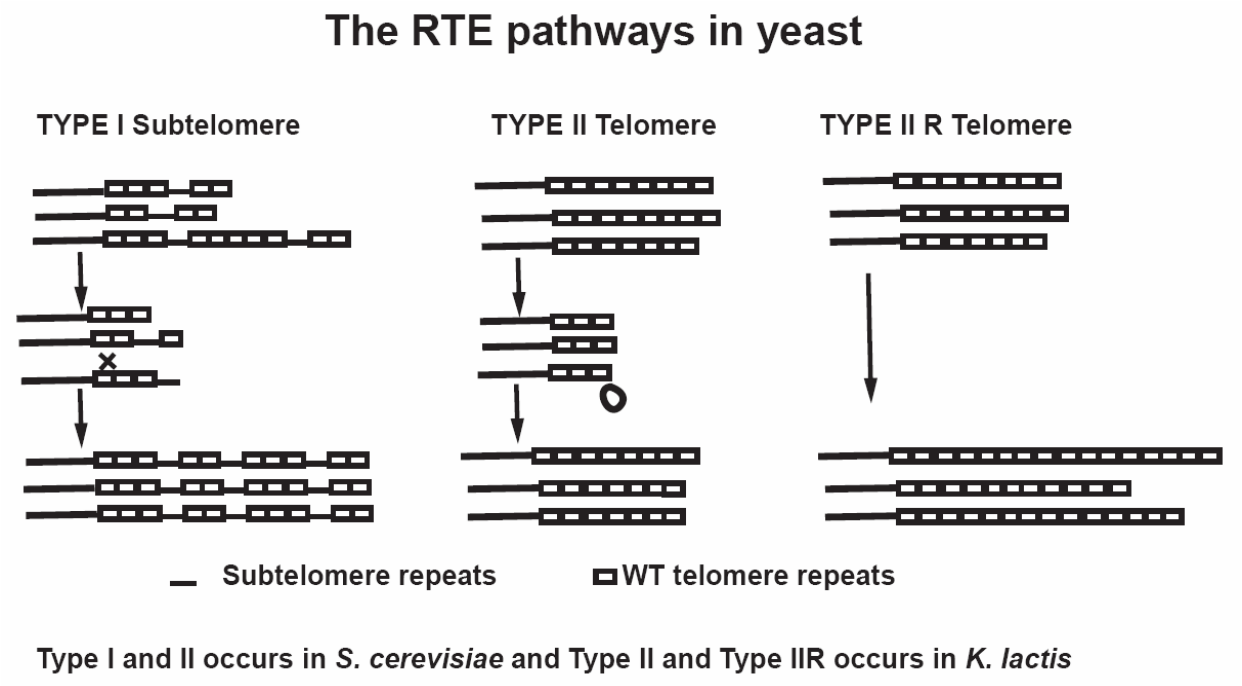
The RTE pathways in yeast
TYPE I Subtelomere
TYPE II Telomere
TYPE II R Telomere
Subtelomere repeats
WT telomere repeats
Type I and II occurs in S. cerevisiae and Type II and Type IIR occurs in K. lactis

Figure 1-2.

Figure 1-3.

Models to explain RTE.

(**A**) The steps in the repair of double-stranded breaks by BIR are shown. The solid and dotted lines represent homologous chromosomes (**B**) The steps in the repair of telomeric ends by rolling circle DNA synthesis are shown. Solid and dotted lines indicates telomeric DNA.

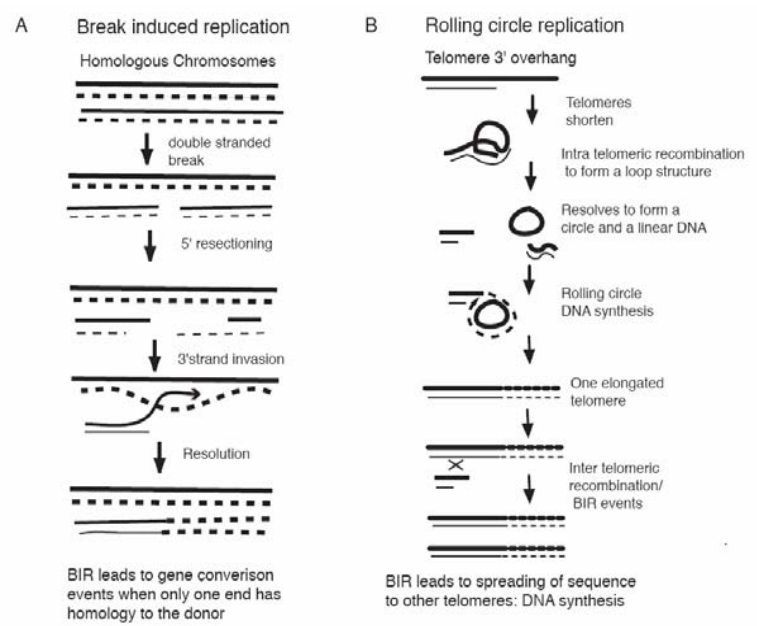
A
Break induced replication
Homologous Chromosomes
double stranded break
5' resectioning
3'strand invasion
Resolution
BIR leads to gene converison events when only one end has homology to the donor
B
Rolling circle replication
Telomere 3' overhang
Telomeres shorten
Intra telomeric recombination to form a loop structure
Resolves to form a circle and a linear DNA
Rolling circle DNA synthesis
One elongated telomere
Inter telomeric recombination/ BIR events
BIR leads to spreading of sequence to other telomeres: DNA synthesis

Figure 1-3

CHAPTER 2

A MUTATION IN THE *STN1* GENE TRIGGERS AN ALTERNATIVE LENGTHENING OF TELOMERE-LIKE RUNAWAY RECOMBINATORIAL TELOMERE ELONGATION AND RAPID DELETION IN YEAST[1]

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[1]Shilpa Iyer, Ashley D. Chadha and Michael J. McEachern. 2005. Molecular and Cellular Biology. Sept. 2005, 25(18). p. 8064-8073.

ABSTRACT

Some human cancer cells achieve immortalization by using a recombinational mechanism termed ALT (alternative lengthening of telomeres). A characteristic feature of ALT cells is the presence of extremely long and heterogeneous telomeres. The molecular mechanism triggering and maintaining this pathway is currently unknown. In *Kluyveromyces lactis*, we have identified a novel allele of the *STN1* gene that produces a runaway ALT-like telomeric phenotype by recombination despite the presence of an active telomerase pathway. Additionally, *stn1-M1* cells are synthetically lethal in combination with *rad52* and display chronic growth and telomere-capping defects including extensive 3'single-stranded telomere DNA and highly elevated sub-telomere gene conversion. Strikingly, *stn1-M1* cells undergo a very high rate of telomere rapid deletion (TRD) upon reintroduction of *STN1*. Our results suggest that *STN1,* the protein protecting the terminal 3' telomere DNA can regulate both ALT and TRD.

INTRODUCTION

Telomeres are the DNA-protein complexes that protect the ends of linear eukaryotic chromosomes (19, 57). They are normally maintained by the reverse transcriptase telomerase, which utilizes a sequence within its RNA component as a template to add new telomere repeats onto the 3' end. Telomerase plays a vital role in immortalization of human cancer cells. Since a majority of human somatic cells lack telomerase activity, expression of telomerase in many cells is sufficient to achieve immortalization, one of the characteristic features of cancer cells (5). Thus, chromosome end protection or 'capping' and telomere length regulation play an important role in preventing cancer and genomic instability in human cells.

How capping is achieved is not fully clear, but a number of proteins binding to the single or double stranded region of the telomeres are known to be involved in regulating this process (9, 19, 42, 57). In the budding yeast *Saccharomyces cerevisiae*, the 3' telomeric termini is protected by Stn1p in association with Ten1p and the single-strand telomere binding protein Cdc13p (11, 18, 24, 25). Recruitment of Stn1p to the telomere by fusion of the DNA binding domain of Cdc13p is sufficient to rescue the lethality of the null mutation of *cdc13* (11, 50). Another yeast telomere protein influencing end protection is Rap1p, which binds to duplex telomere sequences (3) and appears to prevent the occurrence of telomere fusions (42). Similarly in humans cells, single stranded telomere binding protein (POT1), and its interacting protein (PTOP/TINT/PIP1), along with duplex telomeric DNA binding proteins (TRF1 and TRF 2), have been implicated in providing end-protection and contributing to telomere length control (1, 4, 35, 57, 67, 68). Additionally, in a variety of organisms the telomeres have been postulated to form a protective structure, where the 3'end is thought to strand invade into the internal duplex region of the telomere to form a t-loop (10, 26, 44, 48, 60, 61). Whether budding yeast telomeres form t-loops is still unknown, however some evidence indicates that they exist as fold-back structures (14).

Defects in capping can lead to a variety of problems including telomere fusions, disrupted telomere length regulation, elevated recombination near telomeres, degradation of the 5' strand of telomeric DNA, DNA damage checkpoint activation, and premature senescence (2, 19, 42). Many or all of these problems arise from telomeres being recognized as DNA double strand breaks (DSBs). In yeast mutants lacking telomerase, telomeres gradually shorten, leading to a gradual decline in growth rate, cell cycle arrest and cell death from critically short or uncapped telomeres. Rare emergence of post-senescence survivors occurs due to recombinational telomere elongation (RTE) (36, 38, 59). In *S. cerevisiae*, telomere elongation by RTE occurs either by

amplification of the telomeric DNA sequences (type II RTE) or by amplification of sub-telomeric DNA sequences while maintaining short telomeric terminal tracts (type I RTE) (36). However, only the type II RTE pathway has been shown to occur in *K. lactis* (38). The *K. lactis* survivors are proposed to arise through a "roll and spread" mechanism (46). According to this model, a small telomeric DNA circle acts as a template to elongate a single telomere by rolling circle replication. Subsequently, spreading of the sequence from this elongated telomere to other telomeres occurs by gene conversion (62). Additional data has shown that telomeric circles transformed into *K. lactis* strains, promote RTE and that DNA circles as small as ~100 bp can form in a mutant with long dysfunctional telomeres (45, 46, 61).

In a minority of human cancer cells, telomerase activity is absent and the telomeres are maintained by the activation of ALT (7). ALT is thought to involve recombination as a sequence tag introduced in a single telomere in an ALT cell lineage often became copied to additional telomeres (17). ALT is best characterized, in addition to lack of telomerase activity, by extreme telomere length heterogeneity and the presence of ALT-associated promyelocytic leukemia body (APBs) (28). APBs are intra-nuclear structures that contain a number of telomere and recombination specific proteins including TRF1, TRF2, RAD51, RAD52 and the RAD50/MRE11/NBS1 complex (28, 37, 54). The molecular mechanisms triggering ALT are presently unknown.

In addition to elongating telomeres, recombination has also been shown to trigger sudden deletion of telomere sequences in yeast, a process termed telomere rapid deletion (TRD) (33, 37). Telomere truncations have also been observed in other organisms such as *Euplotes, Tetrahymena, Xenopus* and in human ALT cells (13, 32, 51, 64). Whether these truncations involve recombination, is currently unknown.

In this study, we have identified a mutation in the *K. lactis STN1* gene manifesting a striking combination of phenotypes including telomere-capping defects, very long and heterogeneous ALT-like telomeres produced by recombination, and a rapid shortening of all telomeres upon reintroduction of *STN1*.

RESULTS

Identification of a *K. lactis* mutant with extremely long telomeres

In order to identify mutants affecting telomere maintenance in *K. lactis*, we performed a screen for haploid mutants exhibiting abnormal colony morphology by ethyl methane sulphonate (EMS) mutagenesis. Earlier work demonstrated that a rough colony phenotype was characteristic of both telomere deletion and runaway elongation mutants of haploid *K. lactis* strains (38, 39). Approximately 10,000 colonies arising from EMS-treated wild type cells were screened at 30°C, and a total of 345 abnormal colonies were identified. After re-streaking on YPD plates to confirm their phenotypes, these mutants were examined for telomere length defects by Southern analysis. Subsequently, we identified 30 mutant candidates exhibiting varying degrees of mild telomere length defects and two mutants that exhibited dramatic telomere elongation. The telomere and colony phenotype of one of the mutants named M1, is shown in Fig.2-1A, and will be the focus of this study. This mutant exhibited a heterogeneous telomere repeat signal, ranging from limit mobility (>20 kb) to ~100 bp (Fig.1B). The presence of telomere signal below 0.7 kb (the size of the sub-telomere DNA in the smallest *Eco*RI telomere fragment in wild type *K. lactis* strain (47), indicated that a significant amount of telomeric DNA existed in extra-chromosomal form. Further passaging of the M1 mutant revealed that the highly elongated telomeres were

maintained without apparent change over at least ~600 cell divisions (Fig.2-1B). A sub-telomere probe exhibited signal migrating from limit mobility to below 1kb (Fig.2-1C). We conclude that telomeres in the M1 mutant are extremely heterogeneous in length, ranging from short to extremely long. In order to estimate the amount of telomeric DNA in the M1 mutant, we quantitatively analyzed the total telomere hybridization signal in the mutant with respect to the wild type strain by using a Phosphor Imager. The results indicated greater than 10-fold increase in the number of telomere repeats in the M1 mutant. An interesting observation was the appearance of occasional sharp bands visible with the sub-telomeric probe (arrows, Fig.2-1C). These may represent unstable telomere-telomere fusions. Similar unstable sharp bands in certain *K. lactis* long *ter1* template mutants have been shown to be telomere fusions (41).

The elongated telomeres in the M1 mutant appear to be composed entirely of telomeric repeats.

We next investigated if the telomere elongation in the M1 mutant was entirely due to amplification of the telomeric repeat sequences. Genomic DNA was extracted and subjected to digestion with several restriction enzymes with 4 bp recognition sequences. Of the enzymes used, only *Rsa*I has a recognition site within the telomeric repeat of *K. lactis* and was expected to cleave away all the telomeric repeats. Southern hybridization using a telomeric probe showed that all digests except *Rsa*I left the long heterogeneous telomeres intact while bulk genomic DNA was cleaved down to small sizes (compare Fig. 2-2A and 2-2B). In contrast, the sample digested with *Rsa*I showed no telomere signal at high molecular weight and only a faint smear that migrated well below 500 bp (Fig. 2-2B). We conclude that the long heterogeneous telomere fragments in the mutant M1 are due to elongated tracts of telomeric repeats. An interesting

observation in almost all examined restriction digested genomic DNA samples from cells of the M1 mutant was the presence of substantial telomeric hybridization signal (but not bulk chromosomal DNA) in the wells (Figs. 2-1, 2-2 and data not shown). This telomeric signal in wells is resistant to proteinase K (data not shown) and appears to be a general characteristic of *K. lactis* mutants with extremely long telomeres (39, 63).This suggested that telomeric DNA from M1 cells was often present in a tangled form unable to migrate into the gels. Interestingly, the fraction of telomeric DNA in the well increased in the *Taq*I digest, the only digest performed at 65ºC. One possible explanation for this result is that the DNA represents recombination intermediates with complex branched structures and heating at 65ºC further promoted the formation of these structures.

STN1 **complements the** ***M1*** **mutation in** ***K. lactis***

To test if a mutation in a single gene was responsible for the defect seen in the M1 mutant, a segregation analysis was performed. The M1 mutant was mated to a wild type *K. lactis* haploid strain of the opposite mating type and the resulting diploids were sporulated. The results showed that only two of the four spores were viable in most of the fifteen tetrads dissected. In each of the three tetrads where three spores were viable,the extra spore grew to exhibit the rough colony morphology characteristic of the M1 mutant. Each of the rough colonies produced as a consequence of sporulation was analyzed for telomere length by Southern analysis and found to contain long and heterogeneous telomeres similar to those seen in the original M1 mutant (data not shown). These results support the idea that a single gene was responsible for both the telomeric defect and the rough colony phenotype of the M1 mutant. Additionally, it also indicated that this mutation was lethal to a high percentage (90%) of spores. The poor viability

of *stn1-M1* spores was observed with diploids produced by backcrossing and its basis remains unknown.

Subsequent efforts focused on complementing the M1 mutation by transforming a *K. lactis* genomic library plasmid into M1 mutant cells. The transformed cells were incubated at 37°C which was semi-permissive to wild-type *K. lactis*, and exacerbated the growth defects of the M1 mutant. Approximately 20,000 transformants were visually screened for larger colony size and smoother appearance than the average transformed colony. Seven different transformants that continued to show signs of improved growth and colony morphology after re-streaking on YPD plates were then examined for telomere length by Southern analysis. Two of these exhibited a complete loss of the long telomere phenotype. Telomeres in these transformants remained short after additional passaging for maintenance of the library-derived plasmid (Fig. 2-3). However, upon plating these strains on plates containing 5- fluoro-orotic acid (5-FOA), which selected for the loss of the library plasmid, we observed the highly elongated telomeres and rough colony phenotypes resembling the original M1 mutant (Fig. 3). Our results suggested that the plasmids present in the transformants were responsible for rescuing the defect of the M1 mutant. After recovery of these complementing plasmids in *E. coli*, both were found to contain ~ 7.5 kb inserts and had identical restriction fragment patterns upon digestion with *Rsa*I. As expected, re-transformation of both the plasmids into the original M1 mutant was found to completely complement the mutation. One of the plasmids, p72, was selected for further sequencing.

To narrow down the complementing region of plasmid p72, a ~2 kb *Xba*I -*Bspe*I fragment was deleted to create pXB3. pXB3 was also found to fully complement the M1 mutant (data not shown). BLAST analysis of the insert sequence from the pXB3 plasmid revealed two

intact open reading frames. One was identified as *RRP8*, a ribosomal RNA processing protein. The other was identified as *STN1,* based on 28% identity and 47% similarity to the sequence of the Stn1 protein of *S. cerevisiae* (Fig. 4). Further supporting our finding that the *K. lactis* gene was indeed a homologue of *STN1,* the two genes were not only the best matches to each other in their respective genomes but shared the same neighboring genes, *RRP8* and *PDC2* (data not shown). BLASTP searches using the two sequences revealed related protein products from other yeast species, one of which, from *Candida glabrata*, is shown in Fig. 4.

We used genomic DNA from the M1 mutant and its isogenic parent 7B520 for PCR amplification of the *K. lactis STN1* gene. Amplified products were purified and sequenced. Analysis revealed a single base substitution resulting in an isoleucine to lysine change at the amino acid position 79 in the M1 mutant, but not in either the 7B520 parent, or the *STN1* gene in plasmid pXB3. The mutation in *stn1-M1* is present in the region of its interaction with Ten1 (C. Nugent; personal communication). From our results we conclude that the phenotypes of the M1 mutant are due to this mis-sense mutation and we designate this allele *stn1-M1*.

Telomere shortening upon reintroduction of *STN1* is very rapid in *stn1-M1* cells.

Previous work on numerous telomerase RNA gene *(TER1)* template mutants has produced extremely elongated telomeres superficially resembling the telomeres in the *stn1-M1* mutant (31, 39, 56, 63). Re-introduction of a wild type *TER1* gene into these mutants rapidly eliminated the cellular defects and the highly smeared appearance of the telomere fragments, but maintained very long telomeres. Only after hundreds of cell divisions did these long telomeres shorten to more normal lengths. Gradual shortening is expected if telomeric sequence loss occurs primarily from incomplete replication of double stranded DNA ends. It was therefore

surprising that complementation of the *stn1-M1* mutant rapidly shortened all the telomeres to near wild type length without any trace of smears (Fig. 3). To further confirm this rapid sequence loss, we performed mating analysis using *stn1-M1* and *ter1-Acc(19A),* the latter, a long telomere *ter1* template mutant, capable of synthesizing telomeric repeats defective at binding the Rap1 protein (30, 39). Each of these strains was mated to three different wild type strains of opposite mating type and the telomere length from the resulting diploids was examined. Similar to previous published work (Smith and Blackburn 1999), we observed that many telomeres in the *TER1/ter1-Acc (19A)* diploid cells were very long, similar to those of the *ter1-Acc(19A)* parent (Fig. 5A). In striking contrast, the *stn1-M1/STN1* diploid cells had only telomeres of near normal length. This was true for each of the three independent diploids examined over 125 generations. This result confirmed that the long telomeres of the *stn1-M1* mutant were shortened to near normal length within ~30 cell divisions after reintroduction of a single copy of *STN1*.

The long telomeres of *stn1-M1* are generated independently of telomerase

In *S. cerevisiae*, *STN1* has been implicated as a negative regulator of telomerase by preventing Cdc13p- mediated telomerase recruitment (11, 22, 25, 50). Consistent with this, several mutant alleles isolated from different laboratories exhibited elongated telomeres that were formed in a telomerase-dependent manner. We therefore hypothesized that telomere elongation in the *stn1-M1* mutant was mediated by telomerase. To address this, *ter1*-Δ *stn1-M1* double mutants were constructed. Diploid strains constructed by mating between *ter1*-Δ and *stn1-M1* haploid strains were found, as expected, to have telomeres of near normal length (data not shown). Tetrads were dissected from three independent diploid strains. Of the 44 dissected spores, we isolated 5 mutants displaying abnormal colonies and elongated telomeres resembling

the haploid *stn1-M1.* Southern analysis demonstrated that 3 of the 5 mutants were *ter1*-Δ *stn1-M1* double mutants. These mutants did not display the declining growth senescence and survivor colony growth pattern characteristic of *K. lactis ter1*-Δ single mutants, instead they exhibited chronic abnormal growth and colony phenotypes indistinguishable from *stn1-M1* single mutants (data not shown). Consistent with this, no change in telomere length was observed when *stn1-M1 ter1*-Δ cells were passaged over 200 cell divisions. Another *ter1*-Δ *stn1-M1* double mutant was generated by a direct disruption of the *TER1* gene in a *stn1-M1* background (see material and methods). This mutant strain also exhibited highly elongated telomeres and rough colony morphology consistent with results from the mating analysis (Fig. 6A). From our results, we conclude that the telomere lengthening in the *stn1-M1* mutant was independent of telomerase.

Telomerase is active in the *stn1-M1* mutant.

To determine whether telomerase was still active in the *stn1-M1* mutant, the native copy of the telomerase RNA gene *TER1* was substituted with a mutant copy capable of adding telomere repeats with *Apa*LI restriction sites (*TER1-20C (ApaL)*, Underwood et al. 2004). The *stn1-M1 TER1-20C (ApaL)* strain was passaged for ~220-275 generations (11 streaks on YPD plates) and genomic DNA from several passages was digested with *Eco*RI and *Apa*LI to probe for *Apa*LI-containing repeats in the long telomeres of the *stn1-M1* mutant strain. Cleavage with *Apa*LI led to shortening of telomeric fragments that increased with progressive passaging (Fig. 6B). These results indicated the presence of a functional telomerase in the *stn1-M1* mutant.

RAD52 is essential for viability of *stn1-M1* mutants

To assess the role of recombination in telomere maintenance in the *stn1-M1* mutant, we deleted the *RAD52* gene, which is known to be involved in most homologous recombination events, in the *stn1-M1* background. A haploid *rad52 K. lactis* strain was mated to the *stn1-M1* strain and the resulting diploids were sporulated. 23 tetrads from 6 independent diploids were dissected. Of these, 21 tetrads exhibited a ratio of viable to non-viable spores of 2:0 and the other 2 tetrads exhibited a ratio of viable to non-viable spores of 2:1. Visual examination of the non-viable spores revealed growth arrest at 2-4-cell stage. 2-4 cells. Southern hybridization analysis confirmed that the two morphologically abnormal clones were *stn1-M1 RAD52* mutants. Thus no viable *stn1-M1 rad52*Δ isolates were identified by this method. We next performed an additional screen for *stn1-M1 rad52*Δ isolates by random spore analysis from the same parental diploids (see material and methods). Of the 64 colonies examined, 8 rough colonies were identified and analyzed by Southern hybridizations. All of these clones proved to be *stn1-M1 RAD52* single mutants. As expected, ~ 50% of normally growing segregants were *STN1 rad52* mutants. We therefore conclude that the *stn1-M1* mutation is synthetically lethal in combination with the *rad52* gene deletion. In other experiments, we attempted to directly disrupt *RAD52* in mitotically growing *stn1-M1* cells. No *stn1-M1 rad52* double mutants were found from these experiments either.

The *stn1-M1* strain contains long stretches of 3' G-rich single stranded DNA

One of the consequences of telomere uncapping in yeast is the presence of extensive 3' single-stranded overhangs due to excessive degradation of 5' telomeric DNA (6, 20, 49, 63). We therefore analyzed *stn1-M1* and *ter1*-Δ *stn1-M1* mutants by non-denaturing in-gel hybridizations

(15). Undigested genomic DNA from both the strains was electrophoresed on a 0.7% agarose gel. When the gel was hybridized with the C-strand telomere oligonucleotide probe, a very strong signal was observed at limit mobility (>20 kb) and in the wells (Fig. 6C). In contrast, when the same samples were hybridized with the probe derived from the G-strand telomeric DNA, we observed a greatly diminished signal. By equilibrating the signal observed to the control denatured plasmid DNA, we estimate that there is ~20-30 fold increase in single stranded G-strand than single stranded C-strand telomeric DNA in *stn1-M1* and *stn1-M1 ter1-Δ* cells. The strong signal seen with the C-strand telomere probe was largely sensitive to digestion with the 3'-5' single stranded Exonuclease ExoI (Figure 6D). These results are consistent with the *stn1-M1* mutant having a capping defect resulting in large 3' single-stranded overhangs. The ~30% of the single stranded G-strand that was resistant to both 1X and 5X levels of ExoI presumably represents gapped regions of the telomeric DNA. Whether the signal visible with the G-strand telomere probe was from single stranded DNA in the *stn1-M1* samples or due to background hybridization to the large amount of double stranded telomere DNA is unclear.

Telomeres in *stn1-M1* cells exhibit high rates of subtelomeric recombination

We have previously shown that *K. lactis* cells with abnormally short or highly elongated telomeres exhibit highly elevated rates of sub-telomeric recombination (40, 63). We hypothesized that *stn1-M1* cells would exhibit elevated recombination rates near the telomeres because of the importance of *RAD52* gene for their survival. To test this, we performed gene conversion assays to measure the sub-telomeric recombination rates (40). Briefly, telomere DNA tagged with a sub-telomeric *URA3* marker was transformed into *stn1-M1* strain where it replaced a single native telomere. Four transformants that were verified by Southern analysis to contain a

single copy of the *URA3* gene were used for the gene conversion assay (see material and methods). The rates of *URA3* loss due to gene conversion was then measured by plating serial dilutions of the cells on media containing 5-FOA as previously described (McEachern and Iyer 2001). Our results indicate that *URA3* loss was ~5000 fold elevated relative to a wild type *STN1* control (Table1). To date this is the highest rate of sub-telomere gene conversion observed among a variety of *K. lactis* mutants exhibiting dysfunctional telomeres (40, 63).

DISCUSSION

The *stn1-M1* mutant has a telomere cap defect.

The Stn1 protein of *S. cerevisiae* is part of the trimeric complex that binds the single-stranded 3' telomeric overhangs and is known to be involved in both the protective capping function of telomeres and in regulating telomerase's ability to lengthen telomeres (11, 22, 25, 50) . This is the first study of a mutant allele of *STN1* in *K. lactis*. A variety of evidence indicates that the *stn1-M1* mutation causes a defect in the protective capping function of telomeres. The extremely long telomeres of *stn1-M1* cells clearly indicate the presence of a defect in telomere length regulation. The abnormal colony and cellular morphologies of the *stn1-M1* mutant resemble those of early senescing yeast telomerase deletion mutants as well as certain telomerase *TER1* template mutants with extremely long telomeres (38, 39, 56). This suggests that the telomeres of *stn1-M1* cells often trigger DNA damage checkpoints, similar to senescing cells lacking telomerase (11, 22, 25, 50). Another sign of a telomere-capping defect in *stn1-M1* cells is the long tracts of single stranded telomeric DNA, specifically of the 3' G-rich strand, consistent with degradation of the 5' strand of the telomere. Studies in *S. cerevisiae* have shown that single

stranded degradation from the 5' end occurs at double strand breaks and at telomeres with certain capping defects (65). As 3' single-stranded DNA is a potent initiator of homologous recombination, it is not surprising that we also observe highly elevated levels of recombination near the telomeres of *stn1-M1* cells. Additionally, the presence of occasional sharp bands seen with a sub-telomere specific probe (Fig.1B) might suggest that fusions may sometimes occur in *stn1-M1* mutant cells, perhaps only after the loss of telomeric repeats. Overall, our data suggest that Stn1 protects telomeres against initiating homologous recombination events. This protection might occur directly through the interaction of the Stn1/Cdc13/Ten1 complex with telomeric DNA (18) or it might occur indirectly through the ability of Cdc13 and Stn1 to interact with components of DNA polymerase α/primase that leads to synthesis of the C-rich strand at 3' G-strand telomeric overhangs (27, 53).. It is possible that the role of Stn1 is in protecting against telomeric recombination is mediated largely or entirely in the S/G2 phases of the cell cycle, as this is when the 3' overhangs are most pronounced (15) . Also, recent evidence suggests that DSB repair during S/G2 is usually mediated by homologous recombination while repair during G1 is mainly mediated by non-homologous end joining (NHEJ) (19).

Chronic uncapping triggers runaway RTE in *stn1-M1* cells.

Although both *stn1-M1* and *ter1*-Δ mutants can maintain telomeres by recombination, they are very different in important ways. A striking difference is in the extent of telomere elongation between *stn1-M1* and *ter1*-Δ survivor cells. *stn1-M1* cells have telomeres that are many kilobases in length, while *ter1*-Δ survivors seldom have telomeres longer than several hundred base pairs (38). Secondly, *stn1-M1* and *ter1*-Δ cells differ in growth characteristics. *ter1*-Δ cells display progressive decline in growth senescence to a point where most cells are

dead or very poorly growing (38). After RTE has lengthened telomeres, growth is improved with survivors often temporarily displaying growth characteristics indistinguishable from wild type cells. This recovery is followed by irregular cycles of additional senescence and growth improvement brought on by the fluctuating and often very short lengths of the telomeres. In contrast, *stn1-M1* cells exhibit a chronic growth defect reminiscent of moderately senescent *ter1-*Δ cells. This growth defect is stably maintained between different growth passages over long periods of time. The two mutants also differ in their response to deletion of genes involved in homologous recombination. In *stn1-M1*, deletion of *RAD52* leads to immediate lethality. In contrast, in a *ter1-*Δ mutant strain, lethality also occurs, but only after >50 cell divisions. Additionally, we have observed that deletion of *RAD59,* a gene homologous to *RAD52*, kills or severely affect growth of *stn1-M1* cells but does not severely affect growth or the formation of survivors in the *ter1-*Δ background (S. Iyer and M. J. McEachern, unpublished data).

We propose that the differences between *stn1-M1* and *ter1-*Δ cells result from fundamentally different types of telomere capping defects. As shown in the model in Fig. 7, *ter1-*Δ cells can be viewed as having a capping defect that is episodic in nature (Fig.7, left). Recent work in our laboratory has shown that telomeres in *ter1-*Δcells are able to recombine only once they have shortened to less than ~4 repeats (100 bp) in size (62). This predicts that once a telomere is lengthened by RTE to a size appreciably above this length, it effectively becomes recapped and resistant to initiating further recombination events. If all telomeres become lengthened and recapped, the cell can grow normally until one or more telomeres again shorten to below ~100 bp. Telomere length can become kilobases long in ‘type II ‘ survivors of yeast cells lacking telomerase. However, this is thought to be triggered by rolling circle replication

events that generate a long telomere in a single step (34, 46). In this circumstance where uncapping is episodic, RTE is inherently self-limiting.

However, in *stn1-M1* cells, we hypothesize that the capping defect is chronic and partly or entirely independent of the initial length of the telomere (Fig.7, right). Recombination events that produce abnormally long telomeres in *stn1-M1* cells cannot restore normal telomere functionality due to the continued presence of the dysfunctional Stn1 protein. Thus even the longest telomere in *stn1-M1* may be capable of initiating recombinational repair and undergoing RTE.

The RTE producing moderate elongation of telomere repeat tracts in yeast mutants lacking telomerase has been termed 'Type II' (12). Since, the telomere elongation of *stn1-M1* cells appears to consist only of elongated telomeric repeat tracts, it could also be described as type II RTE. However, given the fundamental differences between RTE in *stn1-M1* and *ter1-*Δ cells, especially in terms of telomere length, we propose that RTE observed in *stn1-M1* cells be called Type IIR for producing runaway telomere elongation. We would define type IIR RTE as extreme telomere elongation by recombination resulting from a chronic telomere-capping defect that is largely or entirely independent of telomere length. By this definition, the moderate telomere elongation caused by recombination in *S. cerevisiae cdc13-1* cells under certain conditions might qualify as a less extreme example of type IIR RTE (21, 23) because telomeres of near normal size can become elongated by recombination. Also, as discussed below, the telomere maintenance in human ALT cells is certainly a candidate for being type IIR RTE.

The mechanistic similarities and differences between type II RTE in *ter1-*Δ cells and the type IIR RTE of *stn1-M1* cells still remain to be determined. Accumulating evidence indicates that the type II RTE in *ter1-*Δ cells occurs through a roll and spread mechanism whereby the first

long telomere is generated by copying a telomeric circle of ~100 bp and all other telomeres are elongated by directly or indirectly copying that telomere (45, 46). It is difficult to predict the role of telomeric circles in type IIR RTE. One possibility is that the long telomeres in *stn1-M1* cells could arise from inter-telomeric recombination without the need of DNA circles. However, if relatively large circles are present in *stn1-M1* cells, they could be potent templates for generating long telomeric repeat tracts through rolling circle events. Therefore a reasonable prediction is that telomeric circles are not essential to type IIR RTE but may be important contributors to it.

An interesting feature of the *stn1-M1* mutant (and perhaps a general characteristic of type IIR RTE) is its apparent ability to undergo RTE in the presence of an active telomerase. A question that then arises is why are *stn1-M1 rad52* cells non-viable, if they have can utilize telomerase to maintain chromosome ends? We suggest that the essential role of recombination in *stn1-M1* cells stems from a function that cannot be replaced by telomerase. One possibility is that recombination is needed to repair chromosome ends that have lost all telomeric repeats. The very high loss rate of a subtelomeric *URA3* gene indeed suggests that recombinational repair very frequently involves subtelomeric sequences in *stn1-M1* cells. A second possibility is that recombination is required to antagonize the production of single stranded telomeric DNA. The accumulation of 5-10 kb of single stranded DNA is thought to be sufficient to arrest yeast cell growth (58).The repair DNA synthesis that is initiated from strand invasion or possibly the strand invasion itself might act to counteract the cell cycle arrest signal produced by single stranded DNA.

Is *STN1* a regulator of TRD?

A striking feature of the *stn1-M1* mutant is the rapid loss of its long telomeres upon reintroduction of *STN1* (Fig. 3 and Fig. 5A). This is very different from a number of other instances where abnormally elongated telomeres are introduced into cells that otherwise maintain shorter telomeres (31, 33, 56, 69).(). In these cases, the long telomeres commonly remain long for protracted periods of growth. However, sudden telomere shortening events attributed to processes other than incomplete replication have been observed in a number of circumstances (37). Especially well documented are TRD events that occur in *S. cerevisiae* (8, 33). Most TRD events are *RAD52* and *MRE-11*-dependent and are thought to be intra-strand recombination events resulting in terminal deletions (37). While its underlying mechanism is unclear, the rapid telomere shortening we report here may represent the most extreme example of TRD reported so far.

Both the presence of very long telomeres formed independently of telomerase and the very high rate of sub-telomeric gene conversion indicate that the telomeres in *stn1-M1* mutants undergo very high levels of recombination. It is quite likely that RTE and TRD in *stn1-M1* cells could result from the same recombination pathway initiated at a telomere. Our data suggest that Stn1 acts to inhibit telomeres from engaging in recombination events in general, but also that it differentially affects RTE and TRD. The presence of long telomeres indicates that telomere lengthening predominates over telomere shortening in *stn1-M1* cells. In contrast, reintroduction of *STN1* into *stn1-M1* cells does not simply complement defects caused by the mutant *stn1* gene and freeze telomeres at long sizes as might be expected. Instead, telomere deletion events clearly predominate over RTE events leading to dramatic telomere shortening of all twelve

telomeres. It is not currently known whether these TRD events occur within a single cell division or whether it occurs more gradually over many cell divisions.

How Stn1p influences the extensive TRD events observed undoubtedly depends on the mechanism by which it caps and protects telomeres. The Cdc13p /Stn1p / Ten1p complex bound to the single strand telomeric overhang may prevent the 3' overhang from engaging in any strand invasion into other telomeric repeats. An additional possibility is that the Stn1-complex may affect the outcome of 3' overhangs that are already engaged in an intra-strand invasion, perhaps by binding to the displaced DNA-loop. This might explain how reintroducing *STN1* into *stn1-M1* cells blocks RTE while continuing to permit TRD events in the cell. However, we cannot yet rule out the possibility that the rapid telomere shortening in *stn1-M1* cells arises due to recombination-independent events.

Parallels between *stn1-M1* and human ALT cells

The phenotypes of *stn1-M1* cells are in many ways strikingly similar to mammalian ALT cells. The most obvious similarity between *stn1-M1* and ALT cells is in the very long and heterogeneous telomeres that are produced by recombination. Also the RTE pathways observed in both *stn1-M1* and ALT cells are genetically recessive (51, 52). Another similarity of ALT cells and *stn1-M1* cells is that both exhibit a mixture of healthy and senescent cells (28, 55). This is suggestive of both having chronic telomere capping defects that can often trigger growth arrests. Like *stn1-M1* cells, ALT cells appear to have elevated levels of telomere recombination (17). Additionally, the presence of telomeric DNA, telomeric proteins and a growing list of recombination proteins in ALT associated PML bodies also suggested the importance of recombination in the ALT pathway (28). So far, APBs have not been identified in yeast species.

Other similarities between ALT and *stn1-M1* cells are that both are able to produce telomeric (t-circles) and that RTE can occur in the presence of telomerase (S. Iyer, A. Cesare, E. Basenko, J. Griffith, and M. McEachern, unpublished data). Whether t-circles contribute to RTE in either case is not known. It is currently unclear whether ALT cells, like *stn1-M1* cells, produce at least some telomeres with long 3' overhangs. Rapid telomere shortening reminiscent of TRD, has also been observed in ALT cells (37, 43, 51, 52). The mechanisms triggering such deletion events in ALT and *stn1-M1*-cells still remains to be determined. Thus, both ALT cells and *stn1-M1* cells share many traits consistent with their telomeres having chronic capping defects and being very frequently engaged in recombinational repair.

An obvious possibility suggested by our work is that the phenotype of ALT cells could arise from mutations in genes encoding proteins that are components of the single stranded-DNA binding complex. Although a human homologue of *STN1* has not been yet identified, one of the several proteins interacting with hPOT1 (part of the TRF complex) are potential candidates for being a functional ortholog of *STN1* (35, 57, 67). Another exciting implication of our results arises from the extreme sensitivity of *stn1-M1* to the absence of *RAD52*. This could imply that ALT positive cells may be much more sensitive to a recombination inhibitors than normal cells. We conclude that *stn1-M1* is an excellent model for understanding how telomere-capping defects can trigger both RTE and TRD. Further studies of *stn1-M1* should therefore provide many additional important insights and lead to a better understanding of these mechanisms in yeast and other organisms.

MATERIALS AND METHODS

Yeast media and strain construction.

All *K. lactis* cells used in this study are derivatives of the wild type haploid strain 7B520 (*ura3-1 his2-2 trp1)* (66). The *K. lactis* plasmid genomic library was used to clone the *STN1* gene was previously described (29).

Serial passaging of all the strains was performed by selecting single colonies and allowing them to grow for three days at 30°C on standard rich media containing yeast extract, peptone and dextrose (YPD). The construction of *stn1-M1 ter1-*Δ and *stn1-M1 TER1-20C(ApaL)* double mutants was performed through standard yeast replacement procedures. Replacement of the native *TER1* gene by a *ter1* gene disrupted by *URA3* construct was done by transplacement as described in (38). Replacement of the *TER1* gene by *TER1*-20C(*Apa*L) was done by a plasmid 'loop in –loop out' procedures described in (39). Additionally, the *K. lactis rad52* and *ter1-Δ*7: *URA3* deletion alleles described previously (38), were used for isolating the double mutants strains *stn1-M1 ter1-*Δ and *stn1-M1 rad52*Δ by mating and tetrad dissection.

Random spore analysis was performed on the spores generated by mating of the 7B520 derivative *stn1-M1 ura3 his2 trp1* with the UA24B *(rad52 ade2)* strain. Spores created from the diploid cells of this mating were scooped off the sporulation plate and incubated in 200 μL of 100T Zymolyase (concentration of 0.17 mg/ml 1 M Sorbitol), at 37°C for 10 minutes to digest the ascus sac. The spores were serially diluted in TE (10 mM Tris and 1 mM EDTA), plated on several synthetic complete (SC) plates lacking uracil and adenine and were plated to an approximate cell density of ~200 viable cells per plate and allowed to form relatively large colonies at 30°C so that rough colony morphology of *stn1-M1* mutants could be easily distinguished. Individual colonies selected for further analysis were digested with *Eco*RI and

hybridized to a *RAD52* gene fragment probe to test for the presence of *stn1-M1 rad52* double mutants.

Southerns and non-denaturing in-gel hybridizations.

Yeast genomic DNA was isolated from overnight liquid YPD cultures grown at 30°C fragments were separated on 0.8% agarose gels and electrophoresed at 25V for 15 hours and an additional 35V for 3 hours. They were transferred to HyBond N+ membranes, and hybridization was performed as described previously (Church and Gilbert) at 49°C, with either end-labeled telomere probes (G-probe:Klac1-25 (5'ACGGATTTGATTAGGTATGTGGTGT-3') or (C-probe: Klac25-1 (5'-ACACCACATACCTAATCAAATCCGT-3'). Hybridization of labeled sub-telomere fragment a (~0.6-kb *Eco*RI-*Xba*I from plasmid pAK25ΔB) using a Random Priming Kit (Stratagene) was also carried out at 65°C as described in (Underwood et al. 2004). The membranes were auto-radiographed using Phosphor Imager analysis (Bio-Rad Molecular Imager). In-gel hybridization experiments were performed as described previously (Dionne and Wellinger 1996), using a telomere oligonucleotide as a probe (Klac25-1) with the modifications described in (16, 40, 56, 63). Approximately 3 μg of undigested DNA was electrophoresed through a 0.7% agarose gel and then analyzed using the conditions described in (16, 40, 56, 63).

Mutagenesis.

EMS (ethyl methane sulfonate) mutagenesis was performed using the wild type haploid strain 7B520 (*ura3-1 his2-2 trp1)*. Briefly, 5 ml (1 x 10^8 cells/ml) of cells were treated with 50 μL of EMS and incubated for 2 hours at 30°C. To obtain a 50% survival rate, 1 ml of the culture was removed after every 30 minutes and the reaction was inactivated by the addition of 8ml of

5% sodium thiosulfate. Serial 10 fold dilution of the cells from 60, 90 and 120 minute time points were plated on YPD plates, to a density of 200 viable cells per plate, and allowed to grow at 30° C for three days, so that rough colony morphology could be easily distinguished.

Sub-telomere gene conversion assay.

The gene conversion assay was performed according to the protocols described previously (16, 40, 56, 63). Briefly, one of the native telomeres in the *stn1-M1* mutant strain was replaced by transformation with a ~2.0 kb *Eco*R1 and *Sac*II 'STU' (sub-telomere, *URA3*) fragment containing the *URA3* gene from *S. cerevisiae* inserted into the sub-telomeric sequence of a cloned *K. lactis* telomere. Serially diluted cells of clones containing the 'STU' fragment were plated on SC plates lacking uracil, SC+5-FOA (5- fluoro-orotic acid) and YPD. Measurement of the loss of the *URA3* gene was performed by counting colonies grown on 5-FOA with respect to the total number of colonies grown on YPD and SC plates.

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REFERENCES

1. **Baumann, P., and T. R. Cech.** 2001. Pot1, the putative telomere end-binding protein in fission yeast and humans. Science **292:**1171-5.
2. **Ben-Porath, I., and R. A. Weinberg.** 2004. When cells get stressed: an integrative view of cellular senescence. J Clin Invest **113:**8-13.
3. **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.
4. **Blasco, M. A.** 2004. Carcinogenesis Young Investigator Award. Telomere epigenetics: a higher-order control of telomere length in mammalian cells. Carcinogenesis **25:**1083-7.
5. **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. Science **279:**349-52.
6. **Booth, C., E. Griffith, G. Brady, and D. Lydall.** 2001. Quantitative amplification of single-stranded DNA (QAOS) demonstrates that cdc13-1 mutants generate ssDNA in a telomere to centromere direction. Nucleic Acids Res **29:**4414-22.
7. **Bryan, T. M., A. Englezou, L. Dalla-Pozza, M. A. Dunham, and R. R. Reddel.** 1997. Evidence for an alternative mechanism for maintaining telomere length in human tumors and tumor-derived cell lines. Nat Med **3:**1271-4.
8. **Bucholc, M., Y. Park, and A. J. Lustig.** 2001. Intrachromatid excision of telomeric DNA as a mechanism for telomere size control in Saccharomyces cerevisiae. Mol Cell Biol **21:**6559-73.
9. **Cervantes, R. B., and V. Lundblad.** 2002. Mechanisms of chromosome-end protection. Curr Opin Cell Biol **14:**351-6.

10. **Cesare, A. J., N. Quinney, S. Willcox, D. Subramanian, and J. D. Griffith.** 2003. Telomere looping in P. sativum (common garden pea). Plant J **36:**271-9.

11. **Chandra, A., T. R. Hughes, C. I. Nugent, and V. Lundblad.** 2001. Cdc13 both positively and negatively regulates telomere replication. Genes Dev **15:**404-14.

12. **Chen, Q., A. Ijpma, and C. W. Greider.** 2001. Two survivor pathways that allow growth in the absence of telomerase are generated by distinct telomere recombination events. Mol Cell Biol **21:**1819-27.

13. **Cohen, S., and M. Mechali.** 2002. Formation of extrachromosomal circles from telomeric DNA in Xenopus laevis. EMBO Rep **3:**1168-74.

14. **de Bruin, D., S. M. Kantrow, R. A. Liberatore, and V. A. Zakian.** 2000. Telomere folding is required for the stable maintenance of telomere position effects in yeast. Mol Cell Biol **20:**7991-8000.

15. **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:**13902-7.

16. **DuBois, M. L., Z. W. Haimberger, M. W. McIntosh, and D. E. Gottschling.** 2002. A quantitative assay for telomere protection in Saccharomyces cerevisiae. Genetics **161:**995-1013.

17. **Dunham, M. A., A. A. Neumann, C. L. Fasching, and R. R. Reddel.** 2000. Telomere maintenance by recombination in human cells. Nat Genet **26:**447-50.

18. **Evans, S. K., and V. Lundblad.** 2000. Positive and negative regulation of telomerase access to the telomere. J Cell Sci **113 Pt 19:**3357-64.

19. **Ferreira, M. G., K. M. Miller, and J. P. Cooper.** 2004. Indecent exposure: when telomeres become uncapped. Mol Cell **13:**7-18.

20. **Garvik, B., M. Carson, and L. Hartwell.** 1995. Single-stranded DNA arising at telomeres in cdc13 mutants may constitute a specific signal for the RAD9 checkpoint. Mol Cell Biol **15:**6128-38.

21. **Grandin, N., and M. Charbonneau.** 2003. The Rad51 pathway of telomerase-independent maintenance of telomeres can amplify TG1-3 sequences in yku and cdc13 mutants of Saccharomyces cerevisiae. Mol Cell Biol **23:**3721-34.

22. **Grandin, N., C. Damon, and M. Charbonneau.** 2000. Cdc13 cooperates with the yeast Ku proteins and Stn1 to regulate telomerase recruitment. Mol Cell Biol **20:**8397-408.

23. **Grandin, N., C. Damon, and M. Charbonneau.** 2001. Cdc13 prevents telomere uncapping and Rad50-dependent homologous recombination. Embo J **20:**6127-39.

24. **Grandin, N., C. Damon, and M. Charbonneau.** 2001. Ten1 functions in telomere end protection and length regulation in association with Stn1 and Cdc13. Embo J **20:**1173-83.

25. **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.

26. **Griffith, J. D., L. Comeau, S. Rosenfield, R. M. Stansel, A. Bianchi, H. Moss, and T. de Lange.** 1999. Mammalian telomeres end in a large duplex loop. Cell **97:**503-14.

27. **Grossi, S., A. Puglisi, P. V. Dmitriev, M. Lopes, and D. Shore.** 2004. Pol12, the B subunit of DNA polymerase alpha, functions in both telomere capping and length regulation. Genes Dev **18:**992-1006.

28. **Henson, J. D., A. A. Neumann, T. R. Yeager, and R. R. Reddel.** 2002. Alternative lengthening of telomeres in mammalian cells. Oncogene **21:**598-610.

29. **Heus, J. J., B. J. Zonneveld, H. Y. Steensma, and J. A. Van den Berg.** 1990. Centromeric DNA of Kluyveromyces lactis. Curr Genet **18:**517-22.

30. **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.

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

32. **Larson, D. D., E. A. Spangler, and E. H. Blackburn.** 1987. Dynamics of telomere length variation in Tetrahymena thermophila. Cell **50:**477-83.

33. **Li, B., and A. J. Lustig.** 1996. A novel mechanism for telomere size control in Saccharomyces cerevisiae. Genes Dev **10:**1310-26.

34. **Lin, C. Y., H. H. Chang, K. J. Wu, S. F. Tseng, C. C. Lin, C. P. Lin, and S. C. Teng.** 2005. Extrachromosomal telomeric circles contribute to Rad52-, Rad50-, and polymerase delta-mediated telomere-telomere recombination in Saccharomyces cerevisiae. Eukaryot Cell **4:**327-36.

35. **Liu, D., A. Safari, M. S. O'Connor, D. W. Chan, A. Laegeler, J. Qin, and Z. Songyang.** 2004. PTOP interacts with POT1 and regulates its localization to telomeres. Nat Cell Biol **6:**673-80.

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

37. **Lustig, A. J.** 2003. Clues to catastrophic telomere loss in mammals from yeast telomere rapid deletion. Nat Rev Genet **4:**916-23.

38. **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.

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

40. **McEachern, M. J., and S. Iyer.** 2001. Short telomeres in yeast are highly recombinogenic. Mol Cell **7:**695-704.

41. **McEachern, M. J., S. Iyer, T. B. Fulton, and E. H. Blackburn.** 2000. Telomere fusions caused by mutating the terminal region of telomeric DNA. Proc Natl Acad Sci U S A **97:**11409-14.

42. **McEachern, M. J., A. Krauskopf, and E. H. Blackburn.** 2000. Telomeres and their control. Annu Rev Genet **34:**331-358.

43. **Murnane, J. P., L. Sabatier, B. A. Marder, and W. F. Morgan.** 1994. Telomere dynamics in an immortal human cell line. Embo J **13:**4953-62.

44. **Murti, K. G., and D. M. Prescott.** 1999. Telomeres of polytene chromosomes in a ciliated protozoan terminate in duplex DNA loops. Proc Natl Acad Sci U S A **96:**14436-9.

45. **Natarajan, S., C. Groff-Vindman, and M. J. McEachern.** 2003. Factors influencing the recombinational expansion and spread of telomeric tandem arrays in Kluyveromyces lactis. Eukaryot Cell **2:**1115-27.

46. **Natarajan, S., and M. J. McEachern.** 2002. Recombinational telomere elongation promoted by DNA circles. Mol Cell Biol **22:**4512-21.

47. **Nickles, K., and M. J. McEachern.** 2004. Characterization of Kluyveromyces lactis subtelomeric sequences including a distal element with strong purine/pyrimidine strand bias. Yeast **21:**813-30.

48. **Nikitina, T., and C. L. Woodcock.** 2004. Closed chromatin loops at the ends of chromosomes. J Cell Biol **166:**161-5.

49. **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.

50. **Pennock, E., K. Buckley, and V. Lundblad.** 2001. Cdc13 delivers separate complexes to the telomere for end protection and replication. Cell **104:**387-96.

51. **Perrem, K., T. M. Bryan, A. Englezou, T. Hackl, E. L. Moy, and R. R. Reddel.** 1999. Repression of an alternative mechanism for lengthening of telomeres in somatic cell hybrids. Oncogene **18:**3383-90.

52. **Perrem, K., L. M. Colgin, A. A. Neumann, T. R. Yeager, and R. R. Reddel.** 2001. Coexistence of alternative lengthening of telomeres and telomerase in hTERT-transfected GM847 cells. Mol Cell Biol **21:**3862-75.

53. **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:**1777-88.

54. **Reddel, R. R., T. M. Bryan, and J. P. Murnane.** 1997. Immortalized cells with no detectable telomerase activity. A review. Biochemistry (Mosc) **62:**1254-62.

55. **Rogan, E. M., T. M. Bryan, B. Hukku, K. Maclean, A. C. Chang, E. L. Moy, A. Englezou, S. G. Warneford, L. Dalla-Pozza, and R. R. Reddel.** 1995. Alterations in

p53 and p16INK4 expression and telomere length during spontaneous immortalization of Li-Fraumeni syndrome fibroblasts. Mol Cell Biol **15:**4745-53.

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

57. **Smogorzewska, A., and T. de Lange.** 2004. Regulation of telomerase by telomeric proteins. Annu Rev Biochem **73:**177-208.

58. **Sogo, J. M., M. Lopes, and M. Foiani.** 2002. Fork reversal and ssDNA accumulation at stalled replication forks owing to checkpoint defects. Science **297:**599-602.

59. **Teng, S. C., and V. A. Zakian.** 1999. Telomere-telomere recombination is an efficient bypass pathway for telomere maintenance in Saccharomyces cerevisiae. Mol Cell Biol **19:**8083-93.

60. **Tomaska, L., J. Nosek, A. M. Makhov, A. Pastorakova, and J. D. Griffith.** 2000. Extragenomic double-stranded DNA circles in yeast with linear mitochondrial genomes: potential involvement in telomere maintenance. Nucleic Acids Res **28:**4479-87.

61. **Tomaska, L., S. Willcox, J. Slezakova, J. Nosek, and J. D. Griffith.** 2004. Taz1 binding to a fission yeast model telomere: formation of telomeric loops and higher order structures. J Biol Chem **279:**50764-72.

62. **Topcu, Z., K. Nickles, C. Davis, and M. J. McEachern.** 2005. Abrupt disruption of capping and a single source for recombinationally elongated telomeres in Kluyveromyces lactis. Proc Natl Acad Sci U S A **102:**3348-53.

63. **Underwood, D. H., C. Carroll, and M. J. McEachern.** 2004. Genetic dissection of the Kluyveromyces lactis telomere and evidence for telomere capping defects in TER1 mutants with long telomeres. Eukaryot Cell **3:**369-84.

64. **Vermeesch, J. R., D. Williams, and C. M. Price.** 1993. Telomere processing in Euplotes. Nucleic Acids Res **21:**5366-71.

65. **White, C. I., and J. E. Haber.** 1990. Intermediates of recombination during mating type switching in Saccharomyces cerevisiae. Embo J **9:**663-73.

66. **Wray, L. V., Jr., M. M. Witte, R. C. Dickson, and M. I. Riley.** 1987. Characterization of a positive regulatory gene, LAC9, that controls induction of the lactose-galactose regulon of Kluyveromyces lactis: structural and functional relationships to GAL4 of Saccharomyces cerevisiae. Mol Cell Biol **7:**1111-21.

67. **Ye, J. Z., J. R. Donigian, M. van Overbeek, D. Loayza, Y. Luo, A. N. Krutchinsky, B. T. Chait, and T. de Lange.** 2004. TIN2 binds TRF1 and TRF2 simultaneously and stabilizes the TRF2 complex on telomeres. J Biol Chem **279:**47264-71.

68. **Ye, J. Z., D. Hockemeyer, A. N. Krutchinsky, D. Loayza, S. M. Hooper, B. T. Chait, and T. de Lange.** 2004. POT1-interacting protein PIP1: a telomere length regulator that recruits POT1 to the TIN2/TRF1 complex. Genes Dev **18:**1649-54.

69. **Zhu, L., K. S. Hathcock, P. Hande, P. M. Lansdorp, M. F. Seldin, and R. J. Hodes.** 1998. Telomere length regulation in mice is linked to a novel chromosome locus. Proc Natl Acad Sci U S A **95:**8648-53.

Table 2-1. Elevated levels of recombination near telomeres in *stn1-M1* cells

The sub telomeric gene conversion rates of *stn1-M1* is based on measurements of five independent transformants of *stn1-M1* mutant containing the *URA3* gene inserted near one telomere (see material and methods). The assay for each clone was performed in triplicate. As controls, the values for the wild type *STN1* clones have been cited from previously published results (40). The standard error was calculated as the standard deviation divided by the square root of N, the number of samples assayed for each strain.

***STN1* Allele**	**Gene conversion rate**				**Relative rate**
	Mutation rate	Std. Deviation	Std. Error	N	
STN1	6.70 E- 06	+/- 2.70 E –05	(7.90 E-06)	(12)	1.0
stn1-M1	3.69 E- 02	+/-2. 54 E– 02	(6.87 E-03)	(15)	~ 5500

FIGURES

Figure 2-1.

Identification of a *K. lactis* mutant with extremely long telomeres *(A).* The left panel shows the rough colony morphology of the M1 mutant while the right panel shows the isogenic 7B520 wild type strain. *(B)* Shown here is a Southern Blot of *Eco*RI- digested genomic DNA from the M1mutant hybridized to a probe, Klac1-25, composed of one repeat (25 nucleotide repeat) of the G-strand *K. lactis* telomeric sequence. The individual clone was passaged for 22 streaks (~440-550 cell divisions) and samples were periodically selected for genomic DNA extraction. The wild type sample is shown overexposed for clarity. *(C)* The same blot was stripped and re-probed with sub-telomeric probe. Rare sharp bands marked by white arrows may represent fusions between chromosomes with few or no telomeric repeats. Molecular weight markers (M), shown in the middle are in kilo base pairs (kb).

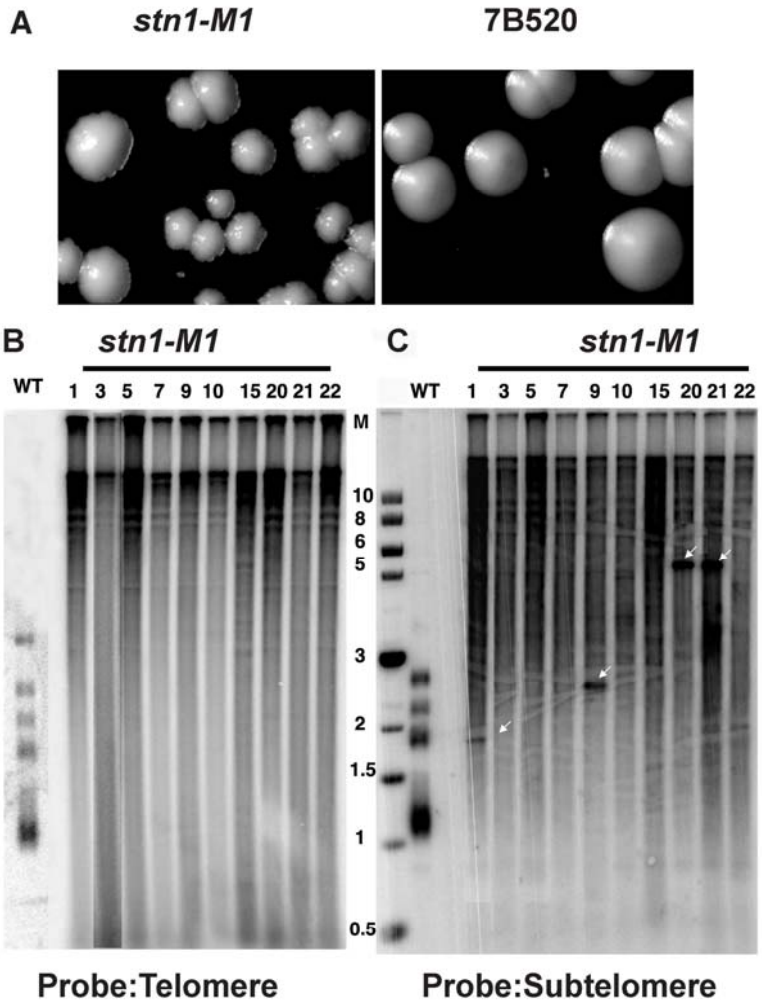
A
stn1-M1
7B520
B
stn1-M1
WT
1 3 5 7 9 10 15 20 21 22
M
10
8
6
5
3
2
1.5
1
0.5
C
stn1-M1
WT
1 3 5 7 9 10 15 20 21 22
Probe:Telomere
Probe:Subtelomere

Figure 2-1

Figure 2-2.

Telomere elongation in the M1 mutant is exclusively due to additional telomeric repeats. *(A)* Ethidium bromide-stained gel of genomic DNA from the 'M1'mutant digested with 8 different restriction enzymes, as labeled. *(B)* The gel in *(A)* was hybridized to the Klac1-25, telomere probe. All the telomeres in lane labeled *Rsa*I have been digested down to ~<50 bp (arrow) as each *K. lactis* telomeric repeat contains an *Rsa*I site.

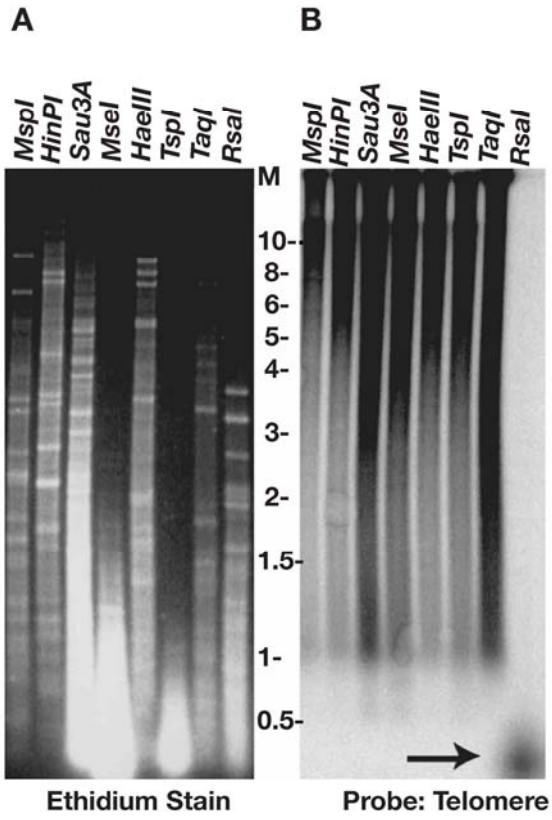
A
B
MspI
HinPI
Sau3A
MseI
HaeIII
TspI
TaqI
RsaI
M
10-
8-
6-
5-
4-
3-
2-
1.5-
1-
0.5-
Ethidium Stain
Probe: Telomere

Figure 2-2

Figure 2-3.

The long telomeres of the M1 mutant are complemented by the *K. lactis STN1* gene. Shown is a Southern blot of *Eco*RI-digested *K. lactis* genomic DNA from the M1 mutant hybridized to a telomere probe. The molecular weight markers (M) are indicated in Kb. The wild type sample (WT) is shown on the first lane, followed by two passages (1-2 streaks) of the M1 mutant. The next three lanes are independent M1 mutant clones shown shortly after transformation with a plasmid containing *STN1* (+p *STN1*). The last five lanes are individual clones of the M1 mutant shortly after losing the *STN1*-containing plasmid (-p *STN1*).

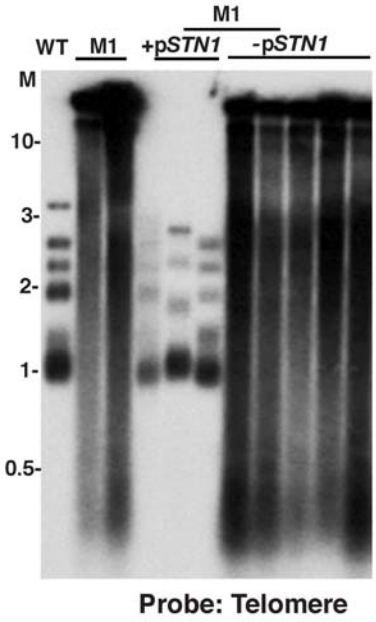

Figure 2-3

Figure 2-4.

Sequence analysis of *K. lactis STN1* gene. This figure represents an alignment of amino acid sequences of *K. lactis* Stn1 with homologues from *S. cerevisiae* (Sc) and *Candida glabrata* (Cg) (genbank accession numbers P_38960 and XP_448655, respectively). The protein sequences were aligned in ClustalW using default values. Identical amino acids are shaded in black and marked with a star. Amino acids showing conserved substitutions are marked with two dots and shaded in grey, and semi-conserved substitutions are shaded in light gray and marked with a single dot. The dark circle indicates the position of the mutation of the *stn1-M1* mutant.

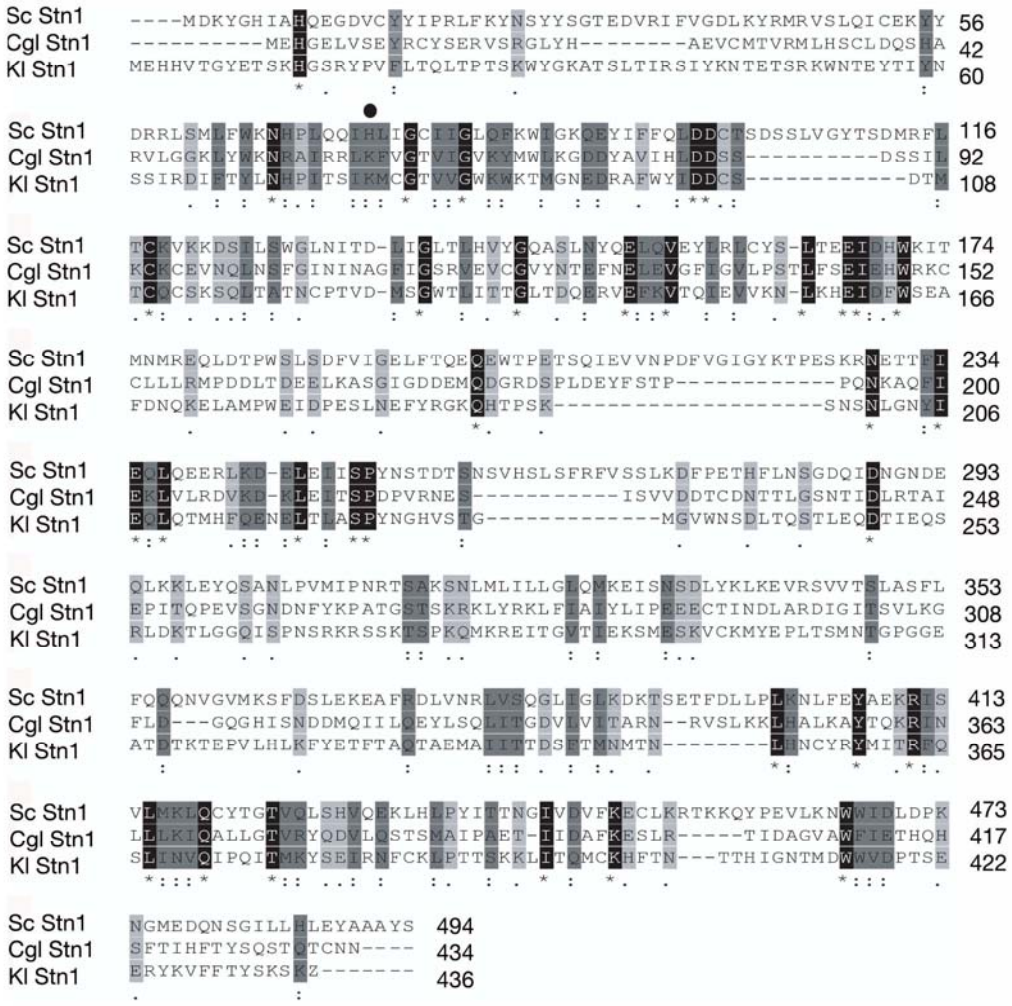

Figure 2-4

Figure 2-5.

Immediate shortening of the telomere occurs upon reintroduction of *STN1*. *Eco*R1-digested genomic DNA of *stn1-M1/STN1* and *ter1-Acc/TER1* diploids hybridized to a telomere probe. The diploids were generated by mating a wild type *K. lactis* strain (material and methods) to the *stn1-M1* strain and to a telomerase *RNA* gene template mutant (*ter1-Acc*) strain. The *stn1-M1/STN1* diploid strain exhibits telomeres of approximately wild type length immediately after mating (within ~30 cell divisions), in contrast to the *ter1-Acc/TER1* diploid, which retains long telomeres. Molecular weight markers shown in the middle are in Kb.

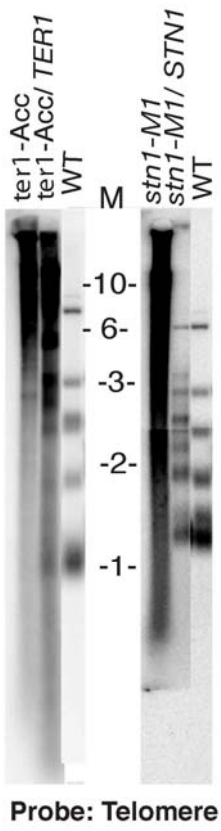
ter1-Acc
ter1-Acc/ TER1
WT
M
stn1-M1
stn1-M1/ STN1
WT
-10-
- 6-
-3-
-2-
-1-
Probe: Telomere

Figure 2-5

Figure 2-6.

***(A)* Telomerase-independent lengthening in *stn1-M1* mutants.** Clones marked 1, 2, 3, 4 are *Eco*R1-digested genomic DNA of *stn1-M1 ter1-*Δ double mutants hybridized to a telomeric G-strand probe (Klac 1-25). Wild type (WT), *stn1-M1* and the parental *stn1-M1/STN1* diploid are also shown. The different intensities of the telomeric signal between the *stn1-M1 ter1-*Δ clones is due to loading differences and possibly also strain differences. Molecular weight markers (M) are in Kb.

***(B)* Telomerase remains active in *stn1-M1* cells**. Shown is a Southern blot of a time course of a clonal lineage of *stn1-M1* containing a silent mutation in the telomerase RNA gene (*TER1-20C (Apa L)*. 1, 3, 5, 7, 9 and 11 represent the number of streaks after introduction of the *TER1-20C (ApaL)* gene, which generates a telomerase that synthesizes repeats with a *Apa*LI site. *Eco*RI (black circle) and *Eco*RI+*Apa*LI (gray circle)-digested genomic DNA of the individual streaks of *stn1-M1 TER1-20C (ApaL)* was hybridized to a G-strand telomere probe (Klac1-25). The last lane shows *Eco*RI-digested genomic DNA from the *stn1-M1 TER1* control. The weaker intensity of the telomeric signal in the *Eco*RI+*Apa*L1 lanes results from telomeric repeats with *Apa*LI sites that are cleaved.

***(C)* Long 3' overhangs in s*tn1-M1* cells.** Shown is a non-denaturing in-gel hybridization of undigested genomic DNA of WT, s*tn1-M1* and *stn1-M1 ter1-*Δ strains. The left half of the panel has been hybridized to a G-strand telomeric probe and the right panel shows the same set of samples probed with a C-strand telomeric probe. Lanes marked 'C' are loading controls, of a denatured plasmid fragment (~3 kb), containing a *K. lactis* telomere. Molecular weight markers (M) are in Kb.

***(D)* Exonuclease I (ExoI) digest of chromosomal DNA from *stn1-M1* cells**. The left half of the

panel is the Ethidium bromide stained gel of genomic DNA from *stn1-M1* cells. The lanes marked ‘1X’ and ‘5X’ represent relative amounts of ExoI used to treat genomic DNA from *stn1-M1* cells. The lane marked ‘U’ shows undigested genomic DNA. The right half of the panel represents the gel from the left panel, hybridized to the C-strand telomere probe.

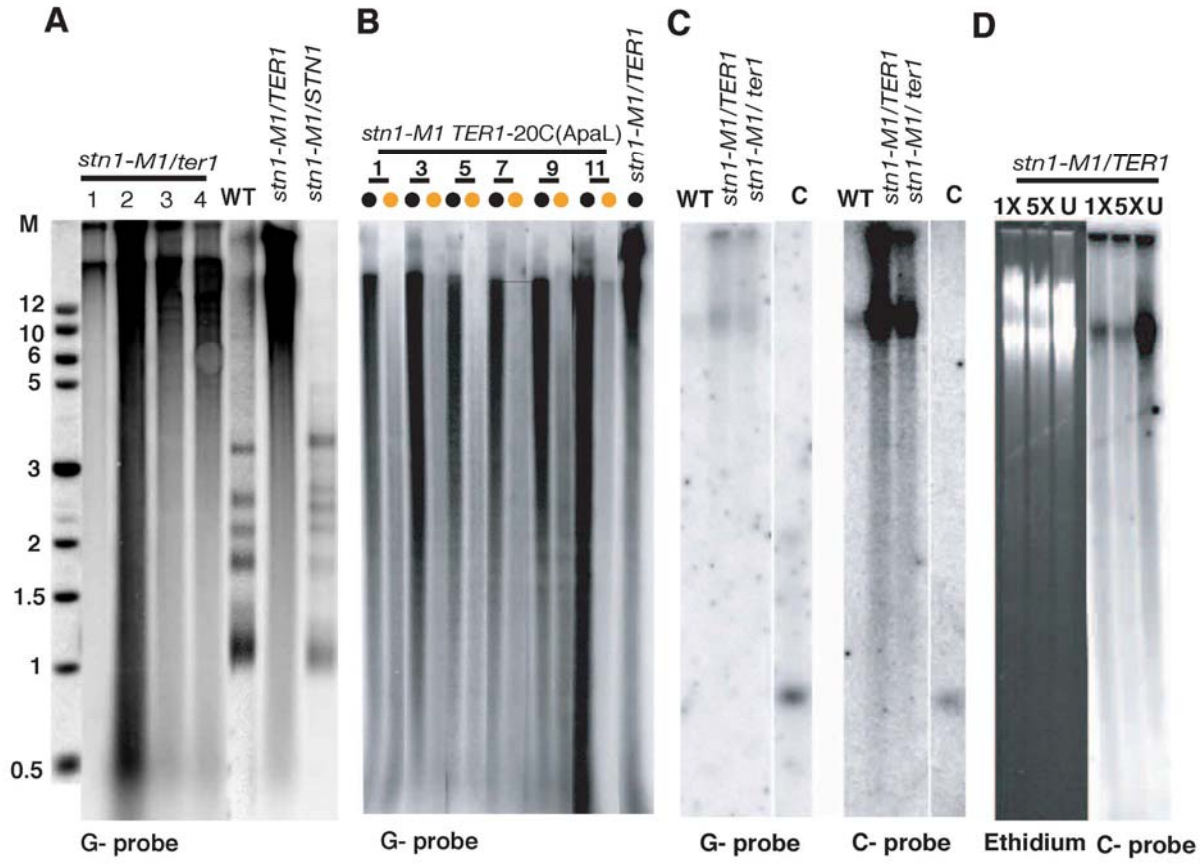
A
B
C
D
stn1-M1/ter1
1 2 3 4 WT
stn1-M1/TER1
stn1-M1/STN1
stn1-M1 TER1-20C(ApaL)
1 3 5 7 9 11
stn1-M1/TER1
WT
stn1-M1/TER1
stn1-M1/ ter1
C
WT
stn1-M1/TER1
stn1-M1/ ter1
C
stn1-M1/TER1
1X 5X U 1X 5X U
M
12
10
6
5
3
2
1.5
1
0.5
G- probe
G- probe
G- probe
C- probe
Ethidium
C- probe

Figure 2-6

Figure 2-7.

Two types of RTE in *K. lactis*. (Left) In the absence of telomerase (*ter1*-Δ), a telomere capping defect producing a state prone to initiating recombination (black star) only occurs once telomeres have dropped below ~100 bp in length, presumably due to the inadequate presence of telomere capping proteins (gray hexagons). Once RTE lengthens telomeres above their critical length, they become recapped and resistant to additional elongation by recombination. The capping-defective telomere state, prone to initiating recombination, is thus episodic and the extent of telomere elongation is limited. (Right) In the *stn1-M1* mutant, a defective capping protein may cause a chronic capping defect that leaves telomeres prone to initiating recombination in a manner largely or entirely independent of telomere length. This produces runaway RTE (Type IIR RTE). See text for details.

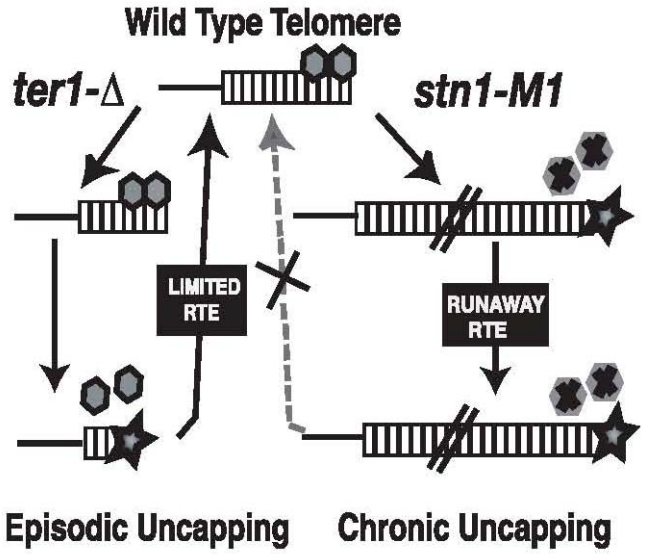
Wild Type Telomere
ter1-Δ
stn1-M1
LIMITED RTE
RUNAWAY RTE
Episodic Uncapping
Chronic Uncapping

Figure 2-7

CHAPTER 3

DETECTION OF DNA CIRCLES IN A YEAST MUTANT WITH TELOMERE CAPPING DEFECTS THAT MAINTAINS TELOMERES USING RECOMBINATION[1]

[1]Shilpa Iyer, Anthony Cesare, Evelina Basenko, Jack D. Griffith and Michael J. McEachern. 2006. Molecular and Cellular Biology. To be submitted.

ABSTRACT

Some human cancer cells are immortalized through a recombination mechanism termed ALT (alternative lengthening of telomeres). A similar recombinational telomere elongation (RTE) pathway occurs in *Kluyveromyces lactis* carrying a point mutation (*stn1-M1*) in the *STN1* gene, a member of the Cdc13p- Stn1p-Ten1p telomere-capping complex. We have labeled this atypical recombination pathway as runaway RTE, which distinguishes it from telomerase deletion mutants that exhibit a modest telomere lengthening RTE phenotype. In addition to long telomeres, *stn1-M1* cells display other phenotypes consistent with a chronic telomere capping defect including rapid telomere deletions, highly elevated subtelomeric recombination and abnormal cells. By employing two-dimensional gel electrophoresis and electron microscopy, we show that *stn1-M1* cells also contain numerous telomere circles of varying sizes and tailed circles resembling rolling circle replication intermediates. Our results imply that telomeric circles in *stn1-M1* cells arise during runaway recombination due to a chronic capping defect at the chromosome ends. These results are significant, because telomeric circles have been postulated to drive RTE in yeast cells lacking telomerase activity. This study has provided the first evidence of such circles in a yeast mutant solely undergoing runaway RTE. Together, our results suggest that *stn1-M1* mutants may serve as an attractive yeast model for understanding the mechanism of both ALT and runaway RTE.

INTRODUCTION

Telomeres are the specialized nucleoprotein structures at the chromosome termini in eukaryotic cells. Telomeric DNA and its associated proteins cap chromosomes ends, masking them from inappropriate double-stranded break (DSB) repair pathways. Telomeres are normally

maintained by the enzyme telomerase which compensates for the gradual loss of telomere repeats at the chromosomal ends through limited DNA synthesis (17). However, homologous recombination (HR) can also provide a mechanism for telomere maintenance in the absence of telomerase or upon the loss of a functional telomere cap. Certain human cancers achieve immortality through a homologous recombination (HR) based mechanism, termed alternative lengthening of telomeres (ALT) (4, 26).

In *S. cerevisiae*, Stn1p appears to be a critical component of a multi-protein complex including Cdc13p and Ten1p that binds and protects the 3' telomeric DNA ends (8). Cdc13p also functions in the recruitment of telomerase to the chromosome ends (2, 5, 6). Furthermore other experiments have demonstrated that recruitment of Stn1p to the telomere can suppress growth and senescence of a null mutant of Cdc13p, indicating its necessity for end protection (20). Genetic and biochemical analyses have also shown that Stn1p interacts with Ten1p *in vivo* (7).

In mammals, a higher order structure termed a telomere loop (t-loop) was discovered that contributed to telomere capping (9). A t-loop is thought to result from an intra telomeric invasion of the 3' overhang in a displacement loop (D-loop) within the duplex telomeric DNA thereby protecting the 3' overhang from the DNA repair machinery. It is not known whether t-loops form at yeast telomeres.

Work in *S. cerevisiae* has shown that an intrachromatid recombination excision mechanism, termed telomere rapid deletion (TRD) truncates elongated telomeres to normal length. This was proposed to occur by the resolution of a t-loop or t-loop like intermediate, possibly leading to the formation of telomeric circles (t -circle) as well as the shortened telomere (12). This results suggests that looped molecules are transiently able to form in yeast, although their role in telomere protection remains unclear. Work in the yeast *K. lactis ter1-16T* mutant

strain, which alters the Rap1 binding site within each telomeric repeat because of a mutation in the template of the telomerase RNA, exhibited long telomeres and phenotypes consistent with telomere capping defects. These included abundant extrachromosomal (ECTR) DNA, excess single-stranded 3' telomeric overhangs and *RAD52*-dependent tiny single and double-stranded telomere circles (10, 23). Human ALT cells have also been shown to exhibit an abundance of ECTR DNA, t-loops and t- circles, as visualized by electron microscopy and by 2D gel electrophoresis (1, 24).

Yeast species mutated for telomerase subunits generate survivors with elongated telomeres through a similar HR-based mechanism called recombinational telomere elongation (RTE) (3, 14-16). In *K. lactis* mutants lacking telomerase, telomeres gradually shorten, leading to cell death from critically short or uncapped telomeres. Rare emergence of post-senescent survivors occurs by amplification of the telomeric DNA sequences (16, 18, 19). The *K. lactis* survivors are proposed to arise through a "roll and spread" mechanism (19). According to this model, a small telomeric DNA circle acts as a template to elongate telomeres by rolling circle replication (rcr). However, t- circles have not been identified in such telomerase deletion cells.

Work in our laboratory on *K. lactis* cells, has identified a novel mutation (*stn1-M1*) in the capping gene *STN1*, that bears a striking resemblance to human ALT cells having extremely long and heterogeneous telomeres from recombination (11). *stn1-M1* cells also exhibit extremely high levels of a TRD–like phenomenon upon introduction of the *STN1* gene (11). In addition, like ALT cells, *stn1-M1* mutants also exhibited many symptoms of chronic capping defects including ECTR DNA, moderate growth senescence and high rates of inappropriate HR at the telomeres (11). The telomere elongation was maintained by recombination despite a functional telomerase

and was labeled as Type IIR, (for runaway), which distinguished it from telomerase deletion mutants that exhibit modest elongation (Type II) (11).

Presented here is the first visual evidence of an investigation of telomeric circles in the *stn1-M1* mutant. The observation of ECTR DNA and an abundance of free telomeric circles of varying sizes in a mutant exhibiting runaway RTE provide further evidence supporting the hypothesis that circles can contribute to RTE. We also show the presence of tailed circles and rcr structures that might be intermediates of the RTE process in *K. lactis*.

RESULTS

Detection of telomere circles in the *stn1-M1* mutant by two-dimensional gel electrophoresis.

K. lactis stn1-M1 cells exhibit telomere dysfunction and elevated recombination similar to what has been observed in human ALT cells [(1, 11), Figure 3-1A]. Previous work on a *K. lactis* telomerase RNA gene (*TER1*) template mutant (*ter1-16T*) produced dysfunctional telomeres and telomere circles (10, 23). This result supported the idea that t-circles could be produced in a yeast mutant with a compromised cap (10). However, telomere elongation in *ter1-16T* could clearly occur independently of recombination, indicating that length regulation was maintained by telomerase (23). Since tiny telomere circles (t-circles) had not yet been identified in telomerase deletion cells or in mutants solely requiring recombination for maintaining telomeres, it was of interest to see if *stn1- M1* cells produced tiny circles.

To test this hypothesis, we first employed two-dimensional (2D) gel electrophoresis and high percentage agarose gels to investigate the low molecular weight (LMW) extra chromosomal DNA in the *stn1-M1* mutant strain. Uncut genomic DNA from a *stn1-M1* strain was examined by

two dimensional chloroquine gel electrophoresis revealing the presence of a ladder that hybridized to both C-strand and G-strand telomeric oligonucleotide probes consistent with the presence of double stranded telomeric repeat circles (Figure 3-1B). This ladder be composed of precise increments, suggesting discreet resolution by recombination pathways of species with integral numbers of 25 bp *K. lactis* telomeric repeats. An arc of DNA material migrating faster than bulk DNA, in the presence of chloroquine and that hybridized only with the C-strand telomeric probe was also present in the uncut genomic DNA from *stn-M1* consistent with the presence of extrachromosomal G-rich single-stranded telomeric DNA. However, unlike previous work with *K. lactis ter1-16T* mutant strain, the single-stranded telomeric DNA material did not migrate as a ladder, but as a smear, suggesting that the single-stranded DNA was not composed of integral numbers of telomeric repeats. In addition, while the faster migrating ECTR material in the second dimension from *ter1-16T* strain was very small, the single-stranded smear in the *stn1-M1* mutant did not migrate as far down in the gel, suggesting the presence of larger ECTR molecules. We were unable to determine if the ECTR material was *RAD52*-dependent as *stn1-M1 rad52*Δ cells were not viable (11).

We next wanted to determine if large circular material was present in *stn1-M1* cells. We, therefore, examined the composition of high molecular weight (HMW) telomere enriched fractions from *stn1-M1* by neutral-neutral 2-D gel electrophoresis (Figure 3-2). Crude nuclei from *K. lactis stn1-M1* cells were isolated and uncut DNA from them was digested with *Alu*I, *Hpa*II and *Nla*III (A/H/N) restriction enzymes. We selected these enzymes specifically due to their lack of non-specific ss-nuclease activity which might cleave ss-DNA at the t-loop junction (personal communication, New England Biolabs, technical support). Greater than 0.5 mg of A/H/N digested DNA was passed through a long gel-filtration chromatography column. Elution

profiles of DNA concentration and specific telomere content indicated that the telomeric DNA preceded the bulk genomic DNA, evidence of sufficient telomere enrichment (Figure 3-3 A, B, C). Digested telomeric DNA fragments (TRFs) from the enriched fractions were separated on 2-D gels(1). In TRFs from *stn1-M1*, a strong circle arc of telomeric material was seen running in conjunction with a circularized Lamda *Hind* III marker as a control (Fig 3-2). These results were consistent with the presence of large circular DNA material even in the HMW fractions of *stn1-M1* samples.

Visualization of telomere circles in the *stn1-M1* mutant by electron microscopy.

To confirm the presence of t-circles in the *stn1-M1* mutant, we next examined the ECTR DNA of the LMW fractions of the *stn-M1* strain using electron microscopy. Undigested samples of *stn1-M1* and *stn1-M1 ter1*Δ genomic DNA were separated on a 0.7% agarose gel and two sets of ECTR DNA were isolated by gel excision procedures. The first gel section was ECTR DNA migrating faster than a 500 bp linear marker, and the second was of ECTR DNA migrating between linear 500 and 3500 bp markers. Subsequent to the excision, the DNA samples from each excised slab, was electroeluted purified and incubated with T4 gene32 ss DNA binding protein and visualized by EM (Figure 3-4).

Although difficulties were encountered in experiments visualizing DNA isolated from the *stn1-M1* and *stn1-M1 ter1*Δ strains, we were able to examine material from the 500 to 3500 bp sample from the *stn1-M1* mutant. In the 500 to 3500 bp gel-excised material, we observed ample ds DNA circles in both the *stn1-M1* (19.5% ± 7.8% of all observed molecules, three experiments) and the *stn1-M1 ter1*Δ strains (30.0% ± 6.8% of all molecules, three experiments). Double stranded circles from the *stn1-M1* and *stn1-M1 ter1*Δ samples, 33 and 21 molecules

respectively, were measured and graphed using the same procedure and classification as above (Table 3-1).

To confirm the presence of larger circles, we employed the high molecular weight (HMW) genomic DNA used for 2-D gels (Fig. 3-2) for electron microscopy. The DNA samples from the (HMW), telomere enriched fractions were prepared for electron microscopy by surface spreading on a denatured protein film (Kleinschmidt preparation). In this method, the DNA was coated with denatured cytochrome *c* which thickened the nucleic acid molecules approximately 10-fold and aiding in inhibiting false positive structures.

We observed a large number of t-circles ranging in size from 0.3 to 31 kb (Fig. 3-5 and Table 3-2). The majority of circles were small, with 73% of the measured circles from the telomeric enriched fractions less than 3 kb in total length, while 11.5 % of the molecules (n=61) were circles above 10 kb. This result was similar to previous experiments in human ALT cells where the bulk of circular molecules were small compared to average telomere size (1).

Visualization of tailed circles in *stn-M1* mutant strain by electron microscopy.

In the course of examining LMW DNA samples from *stn1-M1*, we also visualized unusual structures resembling possible replication intermediates (Fig.3-7 C-E) and several ds-DNA circles with tails of either ds, ss and ds, or only ss DNA (data not shown). These structures were not abundant representing less than 3.5% of the total number of molecules visualized in *stn1-M1* experiments. In some instances, these molecules were stained with the T4 gene 32, at the base of the tail, indicating ss DNA (Figure 3-6 A-C). The average size of the circle + tails shown here was 1389 bp consistent with the size of the DNA molecules isolated. The HMW fraction from *stn1-M1* also contained a few heterogeneous looped molecules, one of which

measured (circle and tail =2.1 kb and 12.6 kb respectively), consistent with the previously characterized t-loop structure, a double-stranded circle with a ds tail (Fig. 3-7 A). Not enough looped molecules were measured in *stn1-M1* cells for accurate size quantitation.

DISCUSSION

Telomeric circles (t-circles) have been postulated to be important contributors to telomere maintenance by RTE in a number of systems. This study has provided the first evidence of telomeric circles in a yeast mutant, *stn1-M1,* which can solely uses RTE to maintain telomeres. Although t-circles have yet to be detected in telomerase deletion cells undergoing type II RTE, considerable evidence now supports the hypothesis that RTE in such mutants proceeds through a roll and spread mechanism. This model postulates that a rolling circle replication event, templated by a small t-circle, generates one long telomere and is followed by spreading of the long sequence to other telomeres through additional recombination events (18, 19, 22). RTE in *S. cerevisiae* telomerase deletion mutants is also thought to occur through a roll and spread mechanism (13). In other cells with DNA ends that undergo RTE, including chromosomes of human ALT cells and the linear mitochondrial DNAs of certain yeasts, t-circles have been shown to be abundant but their role in telomere maintenance is less certain (1, 21, 24). Therefore, the presence of variably sized t-circles in *stn1-M1* mutant strongly suggests that they could be a major factor in elongating telomeres in cells undergoing RTE.

Telomeres normally protect chromosome ends from the DNA repair processes of homologous recombination and non-homologous end joining (NHEJ) and RTE appears to be triggered by different classes of mutants, with a defective telomerase and with a capping gene mutation, that compromise this protective cap. It is highly possible that the frequency of circle

formation in these two main class of mutants could be highly dependent on how often the cell is prone to recombinational repair. Since *stn1-M1* cells are always in a recombination prone state of repair, it is highly probable that they are frequently producing circles, while in telomerase deletion cells, which undergo recombination prone states in an episodic manner, circle production is likely be a less frequent event. The length of the telomeres is probably even more important. The very short telomere of a telomerase *Δ* mutant may be much less likely than the long telomeres of an *stn1-M1* mutant.

The capping defect of *stn1-M1* cells results not only in elongated telomeres but also in other defects including abnormal cell and colony growth, large 3' telomeric overhang and greatly increased rates of both subtelomeric recombination and telomeric truncation events (11). The formation of ECTR, including t-circles, is expected to be another consequence of this capping defect. The t-circles formed in *stn1-M1* cells range from very large (at least 30 kb) to very small (~100 bp) sizes. The presence or absence of telomerase does not appear to appreciably change the profile of t-circles formed in *stn1-M1* cells.

Although the *stn1-M1* mutant displays a similar telomere phenotype to the telomerase RNA template mutant (*ter1-16T*) where t-circles were previously characterized, there are important differences in the nature of t-circles present in each mutant. One difference is that the amount of small ECTR observed in 2-dimensional gels is greater in *ter1-16T* cells. Another difference is that small ECTR from *ter1-16T* contains a much larger percentage of ss t-circles. Single stranded t-circles, specifically of the G-rich telomeric strand, were reported to be ~40% of small t-circles observed by EM in the *ter1-16T* mutant but were <2% of total t-circles in LMW samples from *stn1-M1* cells. Although a relatively high background may have led to an underestimation of very small ss t-circles observed by EM in this study, 2-D analysis confirms

that a high percentage of the *stn1-M1* cells have distinctly fewer small ss t-circles than *ter1-16T* cells (this work and (10). This could stem from differences in how t-circles are made or how they are processed within the two mutants. As Stn1 is a component of the yeast single strand telomeric binding complex (along with Cdc13 and Ten1), a disruption in its function might easily affect the stability of single stranded t-circles that would be expected to bind it. An alternate possibility is that in *stn1-M1* cells, ss-DNA circles form at the same rate as in *ter1-16T*, but are more effectively converted to ds-DNA circles. This might occur because of high rate of strand exchange reactions between ds-linear molecules and ss-circular DNA molecules in *stn1-M1* mutant cells. It will be very worthwhile to test this concept and determine the fate of ss-DNA t-circles between these mutant strains in the absence of the stand exchange protein Rad51.

The precise mechanism of t-circles formation in *stn1-M1* cells or in cells elsewhere, is currently unknown. The leading hypothesis is that they form via a t-loop intermediate whereby the 3' end of the telomere strand invades a more internal region of the same telomeres (12) (Fig. 3-8 C, D). In favor of this possibility, there is a similarity in the size distribution of t-circles with the loop portion of t-loop structures in both human ALT cells and *K. lactis ter1-16T* cells [(1) and Cesare et al, in preparation]. If the initial circular product of a recombination event is partly single stranded, this might lead to formation of both ds and ss t-circles (10). However, other routes can be postulated for the formation of t-circles. The ends of linear extrachromosomal pieces of telomeric DNA, processed to have 3' overhangs, could directly anneal to form a circle (Fig. 3-8 A, B). Alternatively, the same 3' ends might strand invade into a telomere and be extended by a DNA polymerase prior to annealing into a circle. Although t-circle formation is generally dependent on genes involved in recombination (10, 13, 24), we cannot dismiss the possibility that a low percentage of ds t-circle formation might even be due to NHEJ.

The extent to which t-circles contribute to the Type IIR RTE of *stn1-M1* cells is also not known. It is quite possible that the role of t-circles is greater during the initial formation of long telomeres (such as would occur after germination of an *stn1-M1* spore derived from a heterozygous diploid with normal length telomeres), than it is during the subsequent maintenance of the long telomeres over many generations. In this scenario, a fresh *stn1-M1* spore would initially have normal length telomeres with a capping defect making them prone to recombination events. Intratelomeric recombination of telomeres might then produce t-circles of up to ~500 bp in length, consistent with the normal length of a *K. lactis* telomere at a chromosome end. Utilization of such t-circles as templates for rolling circle replication events might then be able to generate telomeres of many kilobases in length in one step. In this model, copying a t-circle might be especially effective at the initial generation of long telomeres by RTE. Once many long telomeres are present in an *stn1-M1* cell, other telomeres could be lengthened by BIR events that copied other chromosome ends in reactions independent of t-circles, leading to runaway recombination. Consistent with this idea, such intertelomeric recombination events have been shown to be very efficient. The sequence of one longer telomere present in a cell is readily copied to all other shorter telomeres during post-senescence survivor formation in a *K. lactis* telomerase deletion strain (22). Thus, t-circles may not be vital to the long-term maintenance of long telomeres in *stn1-M1*, but may play a major role in the initial lengthening of the telomeres. However, the presence of large t-circles in *stn1-M1* cells and the expected ability of these circles to be utilized, would argue that t-circles are likely to at least contribute to the maintenance of long telomeres in *stn1-M1* cells. Consistent with this idea, we observed tailed loops of telomeric DNA from *stn1-M1* cells. These could represent t-circles being utilized conceivably for rolling circle replication events in *stn1-M1* cells.

The Type IIR RTE of *stn1-M1* cells is very similar in many ways to the ALT phenotype of some human cancers and immortal cell lines. In this study, our observation of abundant t-circles of a broad range of sizes only furthers that similarity. Continued investigation of the *stn1-M1* mutant should therefore provide insight into the behavior of ALT cells and their telomeres.

MATERIALS AND METHODS

Yeast strains

The strain 7B520 (*ura3-1 his2-2 trp1*) originally described by Wray and colleagues (25),was used as WT in this study. The *stn1-M1* and *stn1-M1 ter1*Δ strains used here were described previously (11).

Southern Hybridizations

Yeast genomic DNA (cut or uncut) was run on 0.7% Sea Kem LE agarose gel (Cambrex Bio Science Rockland Inc., Rockland, ME) or 4% Gene Pure 3:1 agarose gel (ISC Bioexpress, Kaysville, UT) and then transferred onto Hybond N+ membrane (Amersham Biosciences, Piscataway, NJ). Southern blots were hybridized and washed at 47°C or 50°C with [γ-P^{32}] labeled probes. Probes were either Klac1-25 G-strand telomeric probe (5'-ACGGATTTGATTA GGTATGTGGTGT-3') or the KC25-1 C-stranded telomeric probe (3'-ACACCACATACCTAA TCAAATCCGT-5'). All hybridizations were carried out in the presence of 500 mM Na_2HPO_4 and 7% sodium dodecyl sulfate (SDS) (5). All washes were done in 100 mM Na_2HPO_4 and 2% SDS.

Two dimensional gel electrophoresis for low molecular weight (LMW) extrachromosomal DNA

Genomic DNA, uncut or exonuclease I treated was run at 75V for 6 hours on a 2% non-denaturing Gene Pure 3:1 agarose gel containing 0.6 µg/ml chloroquine. These gels were then soaked in 0.5 X TBE containing 3 µg/ml chloroquine. The gels were rotated 90° and run in the second dimension for 6 hours at 75V. Both dimensions were run in 0.5 X TBE running buffer with chloroquine concentrations equal to that of the gel.

Two dimensional gel electrophoresis for high molecular weight (HMW) genomic DNA

Telomere restriction fragments were separated in the first dimension in a 0.6% Gold agarose (ISC Bio Express, Kaysville, UT) gel in 0.5X TBE (44.5 mM Tris Base, 44.5 mM boric acid, 1 mM EDTA) at 1V/cm for 13.5 hours at RT. The gel was stained with ethidium bromide in 0.5X TBE, de-stained in 0.5X TBE, visualized by UV light and the appropriate lanes excised. The excised slab was arranged in a gel casting tray such that it will be perpendicular to the electrical current. A 1.1% agarose (Invitrogen, Carlsbad, CA) gel in 0.5X TBE containing 300 ng/ml ethidium bromide was poured around the excised slab. Second dimension electrophoresis was carried out at RT in 0.5X TBE containing 300 ng/ml ethidium bromide at 6V/cm for 3 hours. Total DNA was visualized using UV light.

Isolation of low molecular weight (LMW) genomic DNA

Uncut genomic DNA from *stn1-M1* and *stn1-M1 ter1*Δ was run on 0.8% agarose gels at 30 V for 90 min. DNA migrating below between 500-3500 bp linear markers and a 500 bp linear marker was excised from the gel. The DNA was electro-eluted from the gel fragments at 90V for

1 hour while enclosed by 12-14,000 MWCO Spectra/Por dialysis tubing (Spectrum Laboratories Incorporated, Ranch Dominguez, CA). Solutions containing eluted DNA were concentrated by using microcon model YM-10 as directed by manufacturer (Amicon Bioseperations, Raleigh, NC).

Isolation of high molecular weight (HMW) genomic DNA

One liter of *stn1-M1* cells were grown to an OD_{600} an exponential phase of ~1.5. Spheroplasting and isolation of nuclei was performed as described previously (1) with the following modification, the lytic enzyme used was 100μg/ml Zymolyase 100T (Seikagaku). Isolated nuclei were suspended in lysis buffer containing 0.5% SDS and 1mg/ml Proteinase K. The DNA was extracted by phenol/chloroform and precipitated with ethanol. Genomic DNA was digested with *Alu*1, *Hpa* II and *Nla* III (A/H/N) (NEB Beverly MA), at enzyme concentrations of 1U/μg for 2 hours, and supplemented with an equal amount of the enzymes for an additional 2 hours. The DNA was initially treated with Rnase (20 mg/ml) followed by 200μg/ml of proteinase K treatment for 1 hour at 55^0 C in the presence of 0.5% SDS and 10 mM EDTA, then extracted twice with phenol and once with chloroform:isoamyl alcohol (24:1). Following ethanol precipitation, the DNA was suspended in 0.5 mL of 10mM Tris pH 7.6, 0.1 mM EDTA, 0.1% SDS for size fractionation. Greater than 0.5 mg of A/H/N digested DNA from stn1-M1 strains were passed through a long gel-filtration chromatography column. Elution profiles of DNA concentration and specific telomere content indicated that for all preparations, the telomeric DNA preceded the bulk genomic DNA, evidence of sufficient telomere enrichment.

Electron microscopy

Gel isolated low molecular weight *K. lactis* DNA was incubated with 20 µg/ml T4 gene product 32 (gift of Nancy Nossal, NIH, Bethesda MD) for 5 min in a buffer containing 10 mM HEPES pH 7.5 and 1 mM EDTA. The samples were treated with 0.6% gluteraldehyde on ice for 10 min and chromatographed over a 2.5 ml BioGel A-1.5M column (Bio-Rad, Hercules, CA). Fractions containing DNA and DNA-protein complexes were prepared for electron microscopy by adsorption onto negatively-charged carbon-coated grids in the presence of spermadine, followed by dehydration through a series of gradated ethanol washes, air dying, and rotary shadowcasting with tungsten under vacuum. High molecular weight (HMW) DNA samples of *stn1-M1* from the telomere enriched fractions were prepared for electron microscopy by surface spreading on a denatured protein film (Kleinschmidt preparation). In this method, the DNA was coated with denatured cytochrome *c* to thicken the nucleic acid molecule approximately 10 fold and aiding in inhibiting false positive structures. Samples were examined on an FEI Tecnai 12 instrument (Eindhoven, The Netherlands). Images were captured using a Gatan Ultrascan US4000SP digital camera (Gatan, Pleasanton, CA) and molecule dimensions determined using Gatan Digital Micrograph 3.0 software. Images for publication were captured on sheet film, and digitized using ACT-1 software (Nikon, Tokyo, Japan) and a Nikon SMZ1000 stereoscope. Brightness and contrast were adjusted using Adobe Photoshop (Adobe Systems, San Jose, CA).

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REFERENCES

1. **Cesare, A. J., and J. D. Griffith.** 2004. Telomeric DNA in ALT cells is characterized by free telomeric circles and heterogeneous t-loops. Mol Cell Biol **24:**9948-57.

2. **Chandra, A., T. R. Hughes, C. I. Nugent, and V. Lundblad.** 2001. Cdc13 both positively and negatively regulates telomere replication. Genes Dev **15:**404-14.

3. **Chen, Q., A. Ijpma, and C. W. Greider.** 2001. Two survivor pathways that allow growth in the absence of telomerase are generated by distinct telomere recombination events. Mol Cell Biol **21:**1819-27.

4. **Dunham, M. A., A. A. Neumann, C. L. Fasching, and R. R. Reddel.** 2000. Telomere maintenance by recombination in human cells. Nat Genet **26:**447-50.

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

6. **Grandin, N., C. Damon, and M. Charbonneau.** 2000. Cdc13 cooperates with the yeast Ku proteins and Stn1 to regulate telomerase recruitment. Mol Cell Biol **20:**8397-408.

7. **Grandin, N., C. Damon, and M. Charbonneau.** 2001. Cdc13 prevents telomere uncapping and Rad50-dependent homologous recombination. Embo J **20:**6127-39.

8. **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.

9. **Griffith, J. D., L. Comeau, S. Rosenfield, R. M. Stansel, A. Bianchi, H. Moss, and T. de Lange.** 1999. Mammalian telomeres end in a large duplex loop. Cell **97:**503-14.

10. **Groff-Vindman, C., A. J. Cesare, S. Natarajan, J. D. Griffith, and M. J. McEachern.** 2005. Recombination at long mutant telomeres produces tiny single- and double-stranded telomeric circles. Mol Cell Biol **25:**4406-12.

11. **Iyer, S., A. D. Chadha, and M. J. McEachern.** 2005. A mutation in the STN1 gene triggers an alternative lengthening of telomere-like runaway recombinational telomere elongation and rapid deletion in yeast. Mol Cell Biol **25:**8064-73.

12. **Li, B., and A. J. Lustig.** 1996. A novel mechanism for telomere size control in Saccharomyces cerevisiae. Genes Dev **10:**1310-26.

13. **Lin, C. Y., H. H. Chang, K. J. Wu, S. F. Tseng, C. C. Lin, C. P. Lin, and S. C. Teng.** 2005. Extrachromosomal telomeric circles contribute to Rad52-, Rad50-, and polymerase delta-mediated telomere-telomere recombination in Saccharomyces cerevisiae. Eukaryot Cell **4:**327-36.

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

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

16. **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.

17. **McEachern, M. J., A. Krauskopf, and E. H. Blackburn.** 2000. Telomeres and their control. Annu Rev Genet **34:**331-358.

18. **Natarajan, S., C. Groff-Vindman, and M. J. McEachern.** 2003. Factors influencing the recombinational expansion and spread of telomeric tandem arrays in Kluyveromyces lactis. Eukaryot Cell **2:**1115-27.

19. **Natarajan, S., and M. J. McEachern.** 2002. Recombinational telomere elongation promoted by DNA circles. Mol Cell Biol **22:**4512-21.

20. **Pennock, E., K. Buckley, and V. Lundblad.** 2001. Cdc13 delivers separate complexes to the telomere for end protection and replication. Cell **104:**387-96.

21. **Tomaska, L., J. Nosek, A. M. Makhov, A. Pastorakova, and J. D. Griffith.** 2000. Extragenomic double-stranded DNA circles in yeast with linear mitochondrial genomes: potential involvement in telomere maintenance. Nucleic Acids Res **28:**4479-87.

22. **Topcu, Z., K. Nickles, C. Davis, and M. J. McEachern.** 2005. Abrupt disruption of capping and a single source for recombinationally elongated telomeres in Kluyveromyces lactis. Proc Natl Acad Sci U S A **102:**3348-53.

23. **Underwood, D. H., C. Carroll, and M. J. McEachern.** 2004. Genetic dissection of the Kluyveromyces lactis telomere and evidence for telomere capping defects in TER1 mutants with long telomeres. Eukaryot Cell **3:**369-84.

24. **Wang, R. C., A. Smogorzewska, and T. de Lange.** 2004. Homologous recombination generates T-loop-sized deletions at human telomeres. Cell **119:**355-68.

25. **Wray, L. V., Jr., M. M. Witte, R. C. Dickson, and M. I. Riley.** 1987. Characterization of a positive regulatory gene, LAC9, that controls induction of the lactose-galactose regulon of Kluyveromyces lactis: structural and functional relationships to GAL4 of Saccharomyces cerevisiae. Mol Cell Biol **7:**1111-21.

26. **Yeager, T. R., A. A. Neumann, A. Englezou, L. I. Huschtscha, J. R. Noble, and R. R. Reddel.** 1999. Telomerase-negative immortalized human cells contain a novel type of promyelocytic leukemia (PML) body. Cancer Res **59:**4175-9.

FIGURES

Figure 3-1

Two -dimensional gel electrophoresis of LMW telomeric DNA from *stn1-M1*.

A) The *stn1-M1* mutant strain has long heterogeneous telomeres. Shown is a Southern blot of EcoRI digested genomic DNA from WT, *stn1-M1TER1* and *stn1-M1- ter1* exhibiting long telomeres. Restriction fragments were separated on a 0.8% 1D agarose gel, transferred to a nylon membrane, hybridized to C-strand telomere probe and visualized by PhosporImager. B) Low molecular weight (LMW) extra chromosomal telomeric DNA from *stn1-M1* contains ds t-circles and G-rich ss telomeric DNA. Southern blots of 2D 2% agarose gels hybridized to either the G-strand or C-strand telomeric probes indicates the presence of ds telomeric circles in the *stn1-M1* strain. G-rich ss telomeric DNA and ds circles are indicated by white and black arrows respectively.

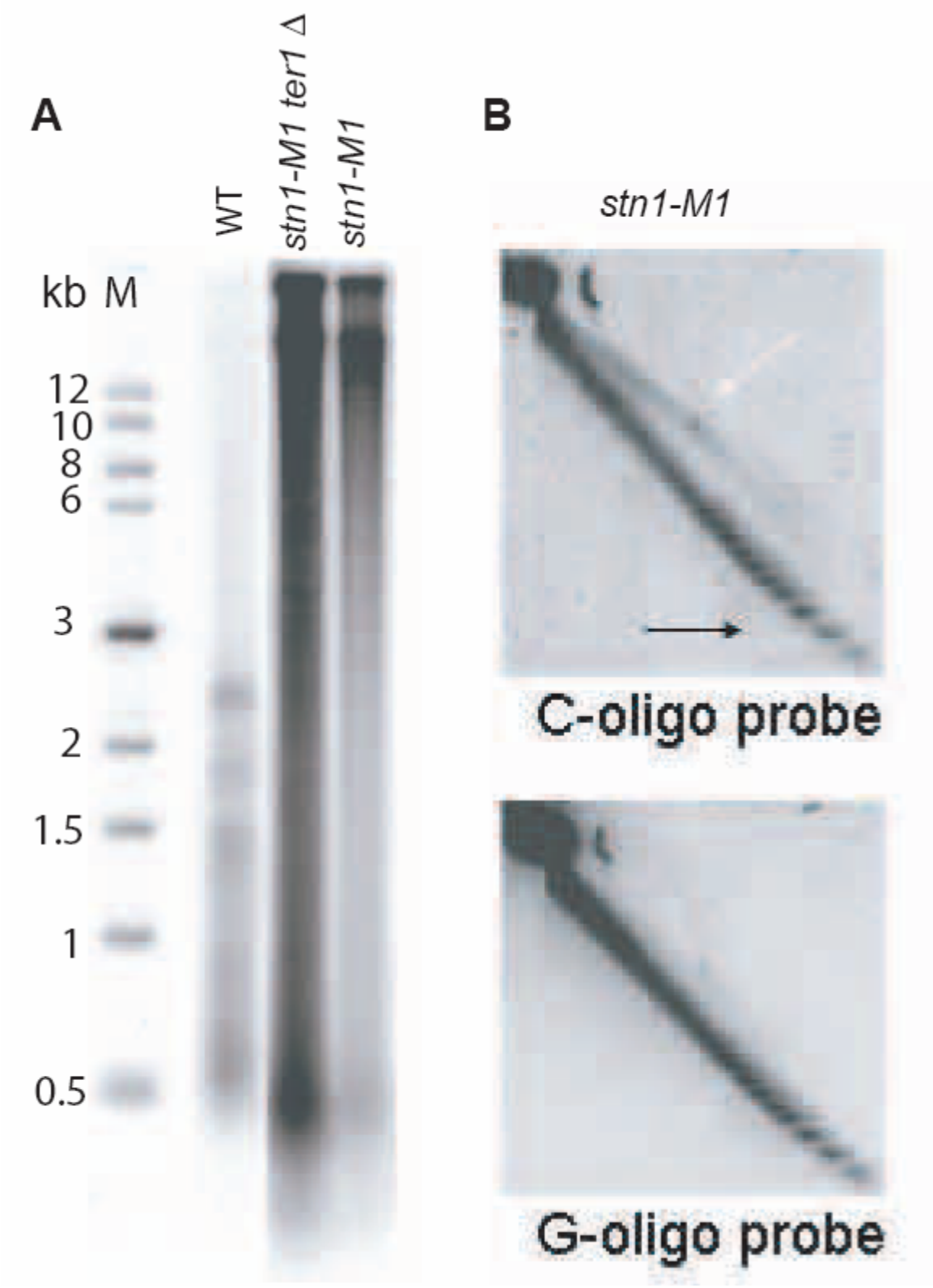
A
WT
stn1-M1 ter1 Δ
stn1-M1
kb M
12
10
8
6
3
2
1.5
1
0.5
B
stn1-M1
C-oligo probe
G-oligo probe

Figure 3-1

Figure 3-2

Two -dimensional gel electrophoresis of HMW DNA from *stn1-M1*.

*Eco*RI digested of high molecular weight (HMW) *stn1-M1* genomic DNA (2ug), and 50 ng of circularized Lambda *Hind*III fragments, were separated in the first dimension in a 0.6% agarose gel, without ethidium bromide (EtBr) at 1V/cm. The excised lane was oriented perpendicular to the electrical current of the second dimension, and cast in a 1.1% agrarose gel containing 300 ng/ml EtBr. The DNA was then separated in a buffer containing 300 ng/ml of EtBr at 6V cm. The gel was dried, probed with a ^{32}P-labeled *K. lactis* C-strand telomere probe, and visualized by PhosphorImager. The gel was subsequently stripped and re-probed with ^{32}P-labelled Lambda *Hind*III fragments. The black, red and blue arrows indicate linear, supercoiled and relaxed circular forms respectively.

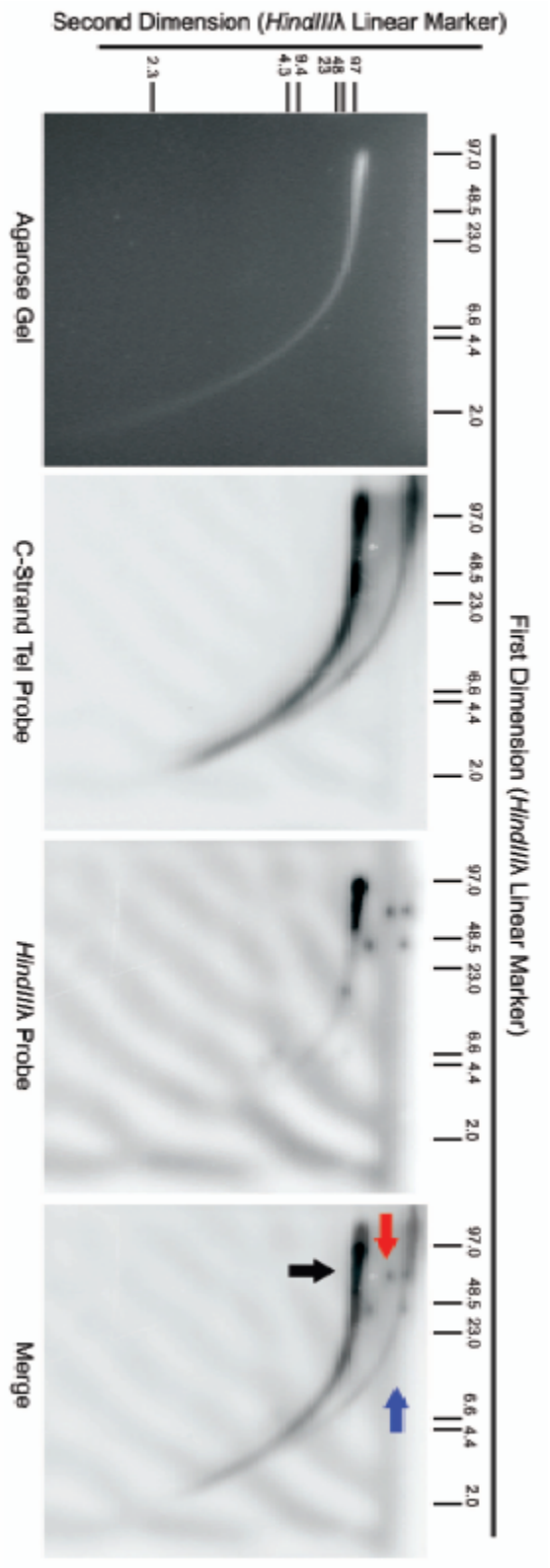
Second Dimension (HindIII λ Linear Marker)
97
48
23
9.4
4.5
2.3
Agarose Gel
C-Strand Tel Probe
HindIII λ Probe
Merge
First Dimension (HindIII λ Linear Marker)
97.0
48.5
23.0
6.6
4.4
2.0

Figure 3-2

Figure 3-3

PFGE measurement and gel-filtration chromatography of telomeric DNA from *K. lactis stn1-M1*

A) Pulsed field gel electrophoresis (PFGE) of DNA from the *stn1-M1* strain. Uncut, *Eco*RI or *Alu*I/*Hae*III/*Mse*I/*Msp*I digested genomic DNA was separated in one dimension by pulsed field gel electrophoresis, and transferred to a nylon membrane by capillary flow following gentle de-purination with HCl. The nylon membrane was probed with a ^{32}P-labeled *K. lactis* C-strand telomere probe and visualized by PhosphorImager. B) Total DNA content and relative telomeric DNA abundance in the eluted fractions following gel filtration chromatography of *stn1-M1 Alu*I/*Hpa*II/*Nla*III digested genomic DNA. DNA content was determined by measuring optical density at 260 nm (OD260), with the scale shown on the left hand side of the profile. Relative telomere abundance was determined by quantitation of the telomere signal from the slot blot of eluted fractions using a PhosphorImager. C) Slot blot analysis of eluted fractions following gel filtration chromatography of *stn1-M1* AluI/*Hpa*II/*Nla*III digested genomic DNA. DNA (50 ng) was transferred to a nylon membrane, probed with a [γ-^{32}P] radio labeled *K. lactis* C-strand telomere oligonucleotide, washed and detected by PhosphorImager. 5 μg of *Alu*I/*Hpa*II/*Nla*III digested *stn1-M1* genomic DNA was used as a load control.

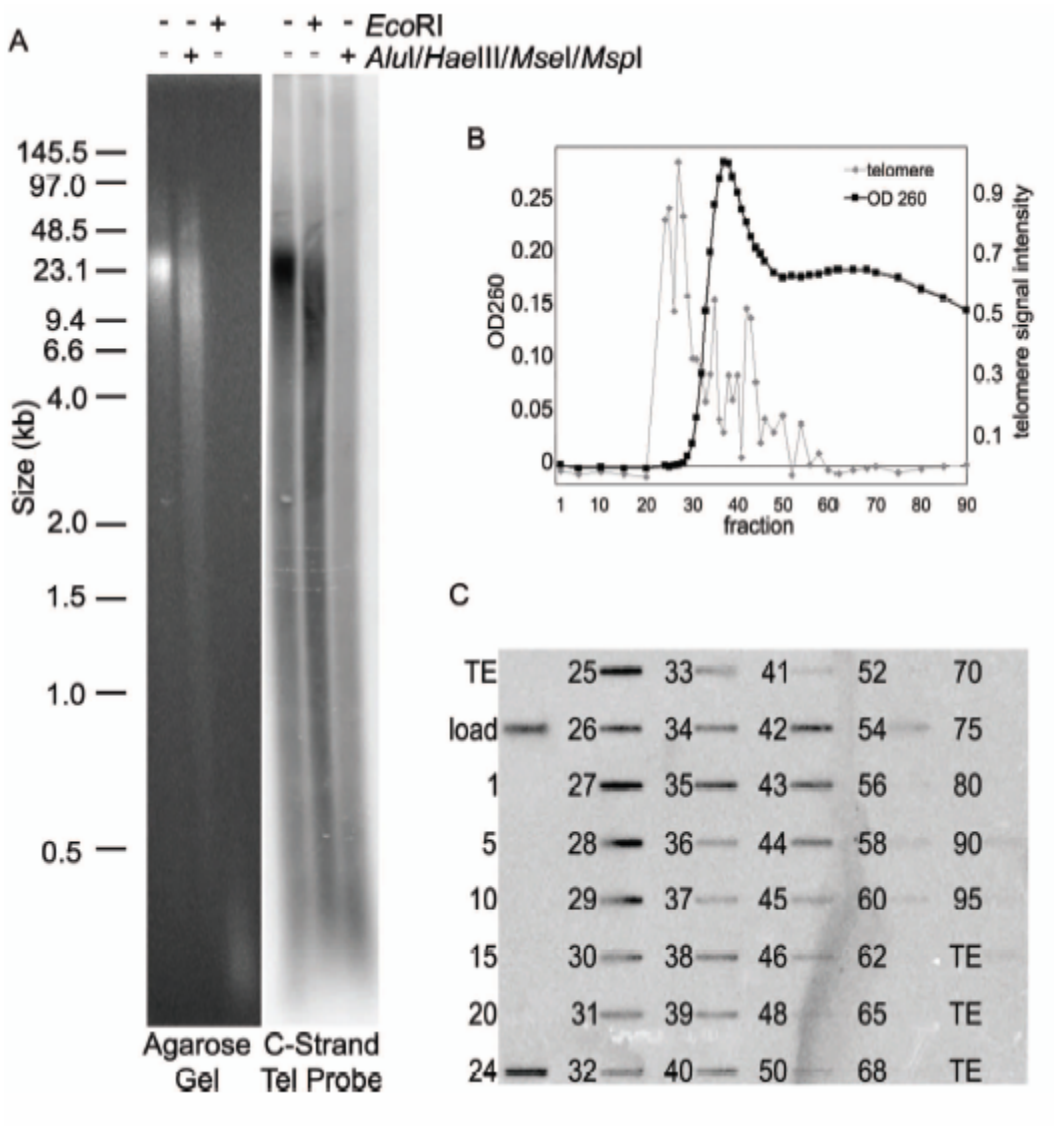
A
- - + - + - EcoRI
- + - - - + AluI/HaeIII/MseI/MspI
145.5
97.0
48.5
23.1
9.4
6.6
4.0
2.0
1.5
1.0
0.5
Size (kb)
Agarose Gel
C-Strand Tel Probe
B
telomere
OD 260
OD260
telomere signal intensity
fraction
C
TE load
1
5
10
15
20
24
25 26 27 28 29 30 31 32
33 34 35 36 37 38 39 40
41 42 43 44 45 46 48 50
52 54 56 58 60 62 65 68
70 75 80 90 95 TE TE TE

Figure 3-3

Figure 3-4.

Visualization of DNA circles from LMW fractions prepared from *stn1-M1 TER1* and *stn1-M1 ter1* cells.

A-F) Electron micrographs of double strand DNA circles. Circle sizes are ~1300, ~420, ~200, ~200 and ~250, ~180, and ~200 bp respectively for A-F. Samples for electron microscopy were prepared by directly mounted onto thin carbon coated coils and rotary shadow cast with tungsten. Shown in negative contrast. Bar is equivalent to 250 bp.

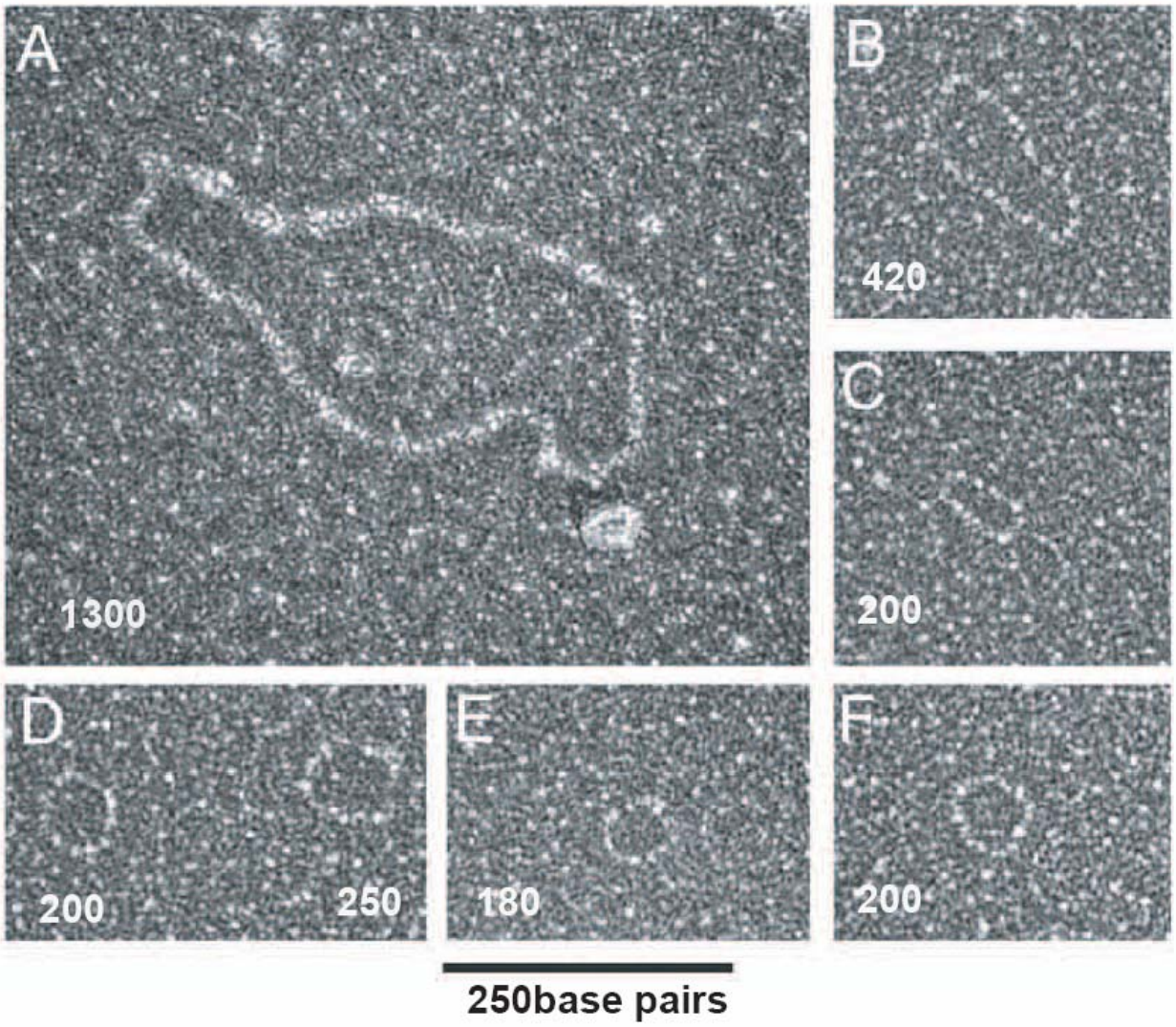

Figure 3-4

Figure 3-5

Size distribution of DNA circles from LMW fractions from stn1-*M1 TER1* and *stn1-M1 ter1*cells.

Measured ds circles observed in ECTR DNA isolated from *stn1-M1* (n = 33) and *stn1-M1 ter1*Δ (n = 21) cells. Bars represent circles ranging from 12.5 bp/nt above and below the size indicated.

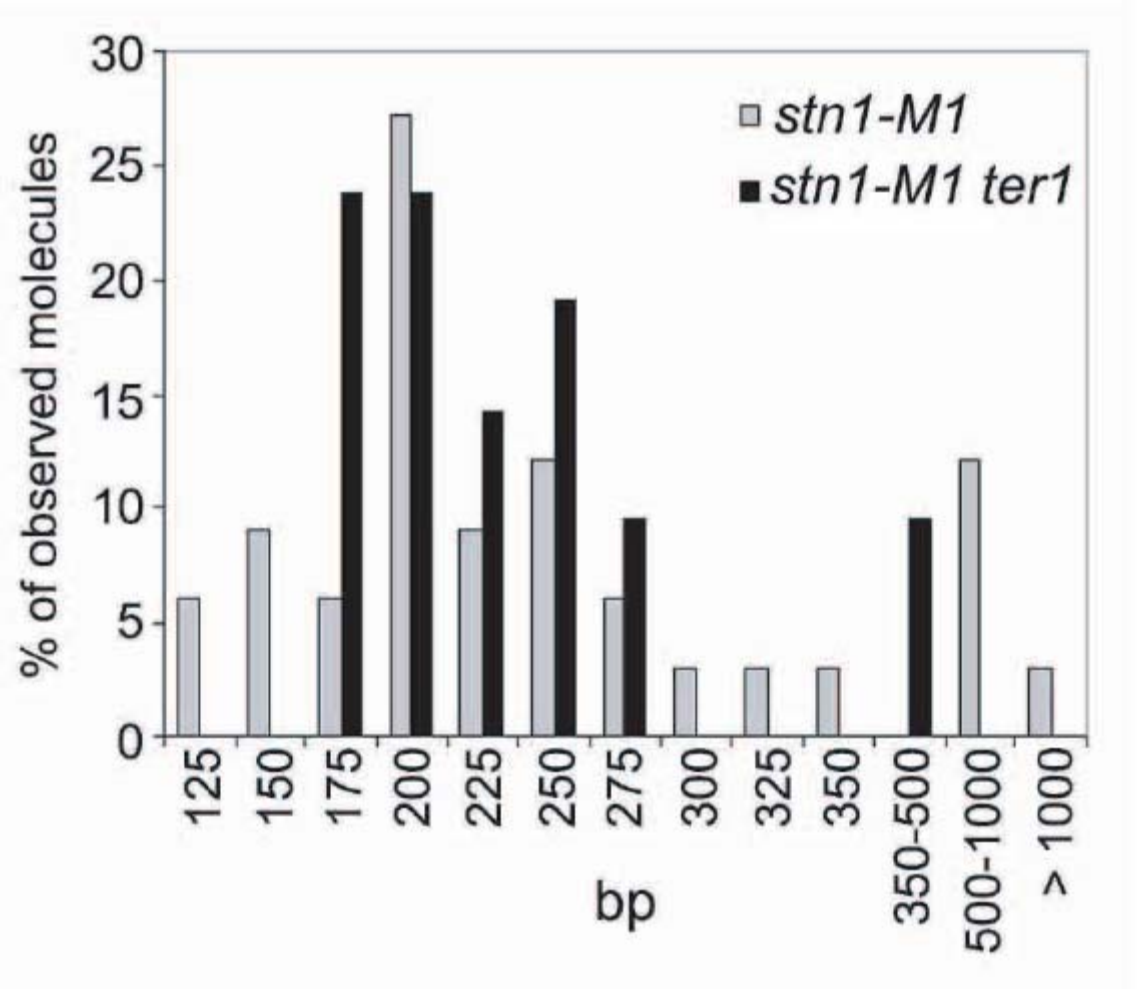
30
25
20
15
10
5
0
% of observed molecules
stn1-M1
stn1-M1 ter1
125
150
175
200
225
250
275
300
325
350
350-500
500-1000
> 1000
bp

Figure 3-5

Figure 3-6

Visualization of DNA circles from HMW DNA from *stn1-M1*cells.

A-E) Electron micrographs of DNA circles observed in the telomere enriched fractions from *stn1-M1* cells. Circle lengths are ~15.7, ~12.1, ~3.1, ~1.2 and ~1.2, and ~0.9 kb for fractions A-E respectively. Note there is a 800 bp linear fragment in the lower right portion of panel A. DNA was prepared for EM by surface spreading with cytochrome *c*, absorbing to a parlodian covered copper EM grid, and rotary shadowcasting with a platinum-palladium amalgam (Kleinschmidt method). Shown in negative contrast. Bar is equivalent to 1.5 kb.

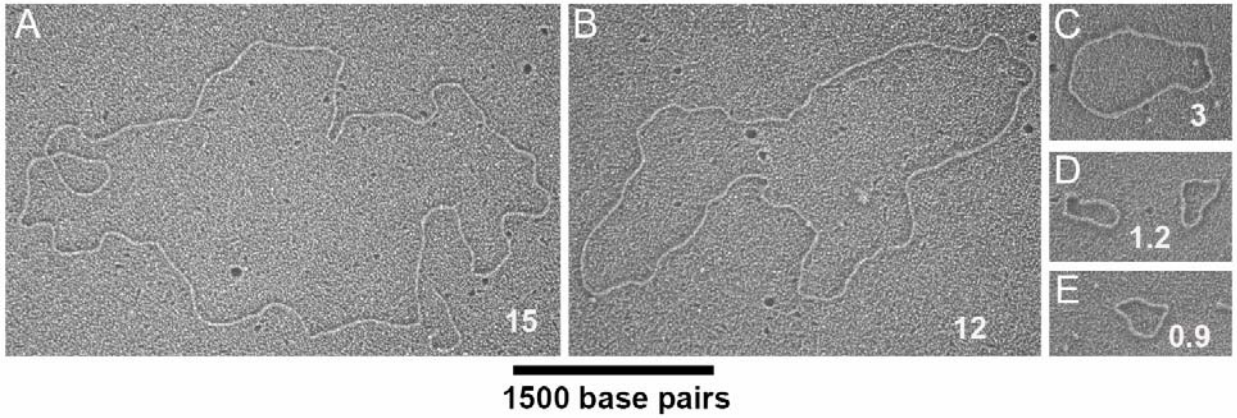

Figure 3-6

Figure 3-7

Size distribution of DNA circles in HMW DNA from *stn1-M1* cells.

Distribution of circle size as a percentage of molecules scored in the telomeric enriched fractions (26, 27, and 28) from non-crosslinked DNA from *stn1-M1* cells (n = 61). Bars (in grey and grey checks) represent DNA-circles ranging from 12.5 bp/nt above and below the size indicated. The black bar in all size fractions is the mean distribution of the total number of observed molecules from all three fractions.

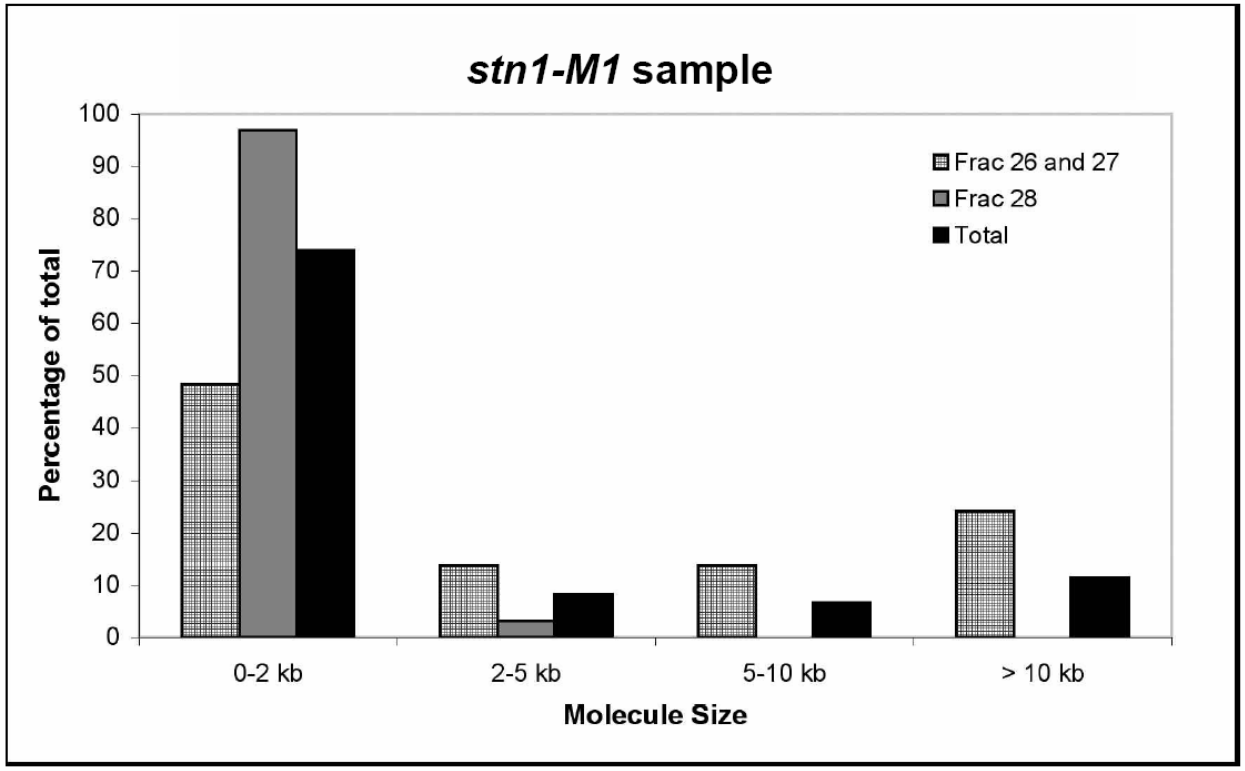
stn1-M1 sample
Percentage of total
100
90
80
70
60
50
40
30
20
10
0
Frac 26 and 27
Frac 28
Total
0-2 kb
2-5 kb
5-10 kb
> 10 kb
Molecule Size

Figure 3-7

Figure 3-8

Visualization of tailed circles in *stn1-M1 TER1* and *stn1-M1 ter1* cells.

A-B) Electron micrographs of tailed circular DNA structures. Circular and tail portions of molecules shown in A-B are: ~420 and 1300, and ~250 and 1000 bp respectively. C) Electron micrograph of a tailed circle structure with a ds and ss DNA tail. The DNA was incubated with T4 gene 32 protein, gluteraldehyde crosslinked and prepared for EM as described in Figure 3-2. The loop portion of the molecule shown in C is ~400 bp and the total length of the single stranded and double stranded tail is equivalent to ~800 bp. Bar is equivalent to 250 bp.

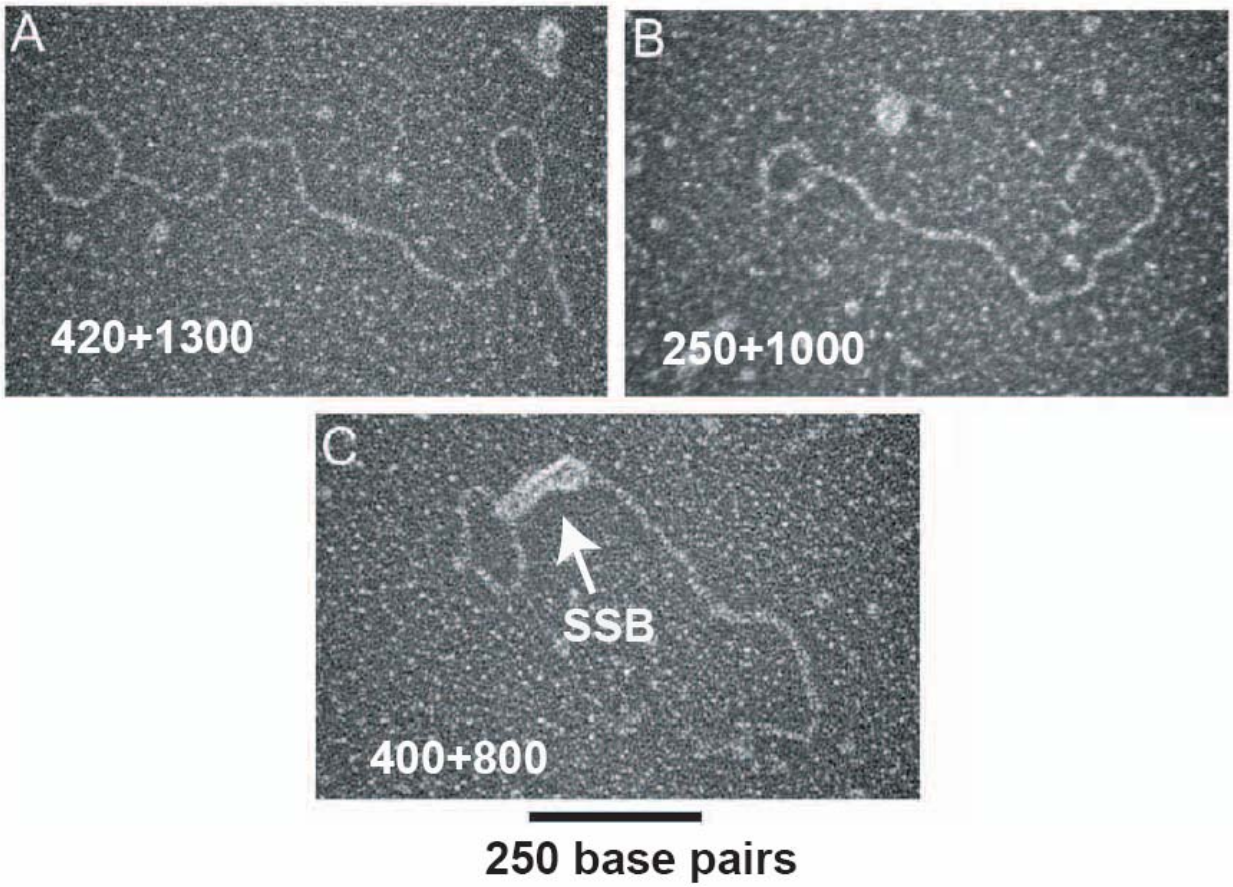

Figure 3-8

Figure 3-9

Other structures in the DNA fractions from *stn1-M1* cells.

A) An example of a tailed-circular DNA molecule from *stn1-M1* observed following telomeric DNA enrichment by gel filtration chromatography of *Alu*I/*Hpa*II/*Nla*III digested genomic DNA. The circle and tail contours of the molecule are 11.2 and 2.2 kb respectively. DNA was prepared for EM by the Kleinschmidt method as described in Figure 3-7. Shown in negative contrast. Bar is equivalent to 1.0 kb. B-E) Electron micrographs of complex DNA structures. Examples of different molecules observed in LMW fraction from *stn1-M1* cells. DNA preparation for EM has been described in Figure 3-2. B) Shown here is a double stranded circular DNA structure with a ds short tail C) This represents a partly single stranded branched 'Y' molecule. The ss branch has been coated with T4 gene 32 protein gluteraldehyde crosslinked and prepared for EM, as described in Figure 3-7. D) Electron Micrograph of a possible double Holliday junction intermediate. E) A possible replicating structure. Double stranded DNA has been folded to form a complex structure with a single-stranded fork and a ds DNA tail. The DNA was incubated with T4 gene 32 protein and prepared for EM. Bar is equivalent to 250 bp.

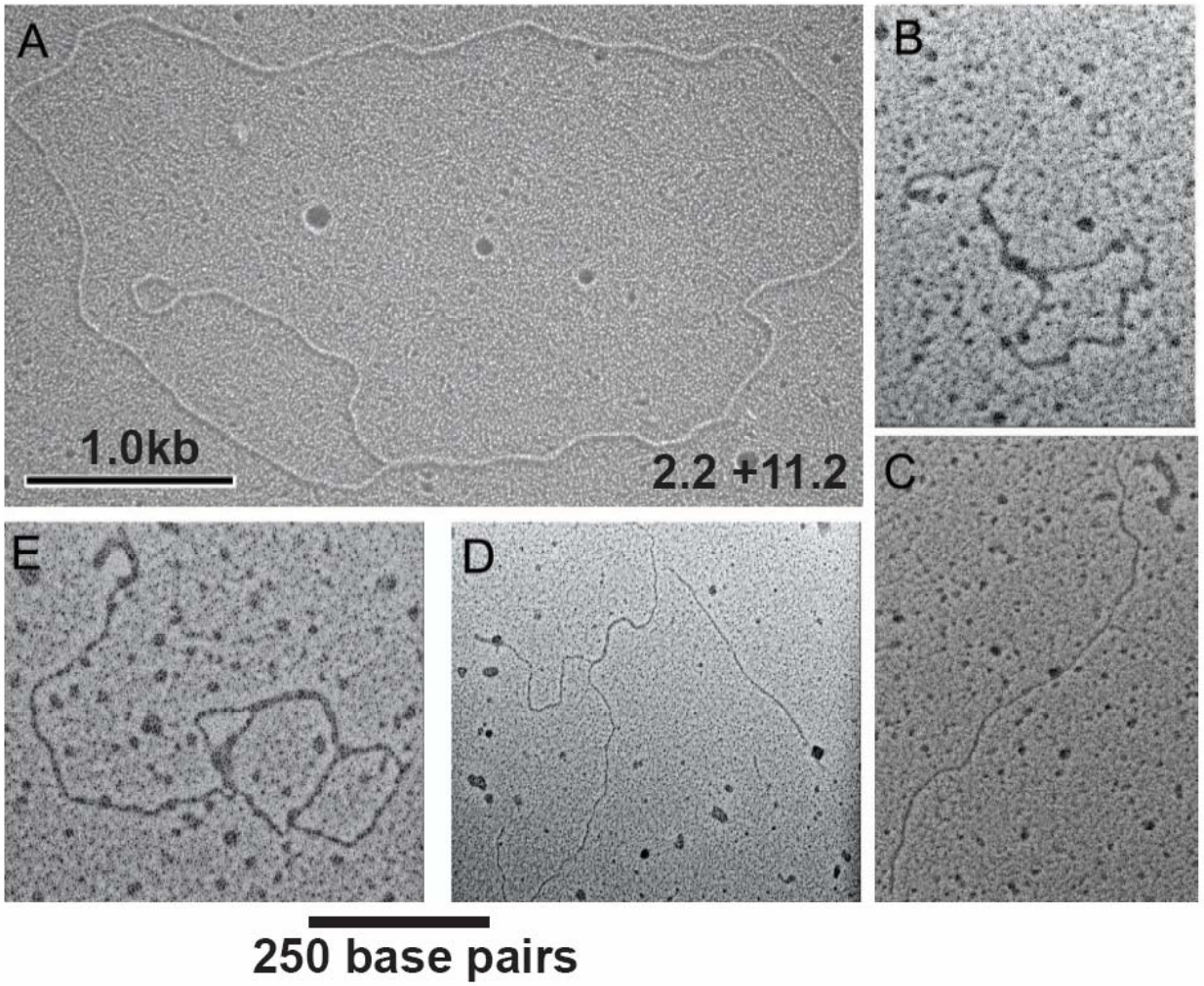

Figure 3-9

Figure 3-10.

Models showing pathways to create and process t-circles.

Resolution of a folded structure by intramolecular deletion events lead to formation of partially ds-DNA circles (A) and ds-linear DNA (B). Partially ds-DNA circles (A) can be processed by either synthesis to form ds circle (D) or degradation to form ss-circle (E). Dark circles indicate G-strand while light circle indicate C-strand. Dotted lines indicate synthesis.

ds-linear DNA (B) can be processed by either end-annealing to form ds-circle (C) or strand-exchange with ss-circle to form ds-circle (D). 3'- strand invasion of ds-circle (F) to form long telomere (G), by rolling circle mechanism. (H) Intermolecular recombination by BIR events to form many long telomeres.

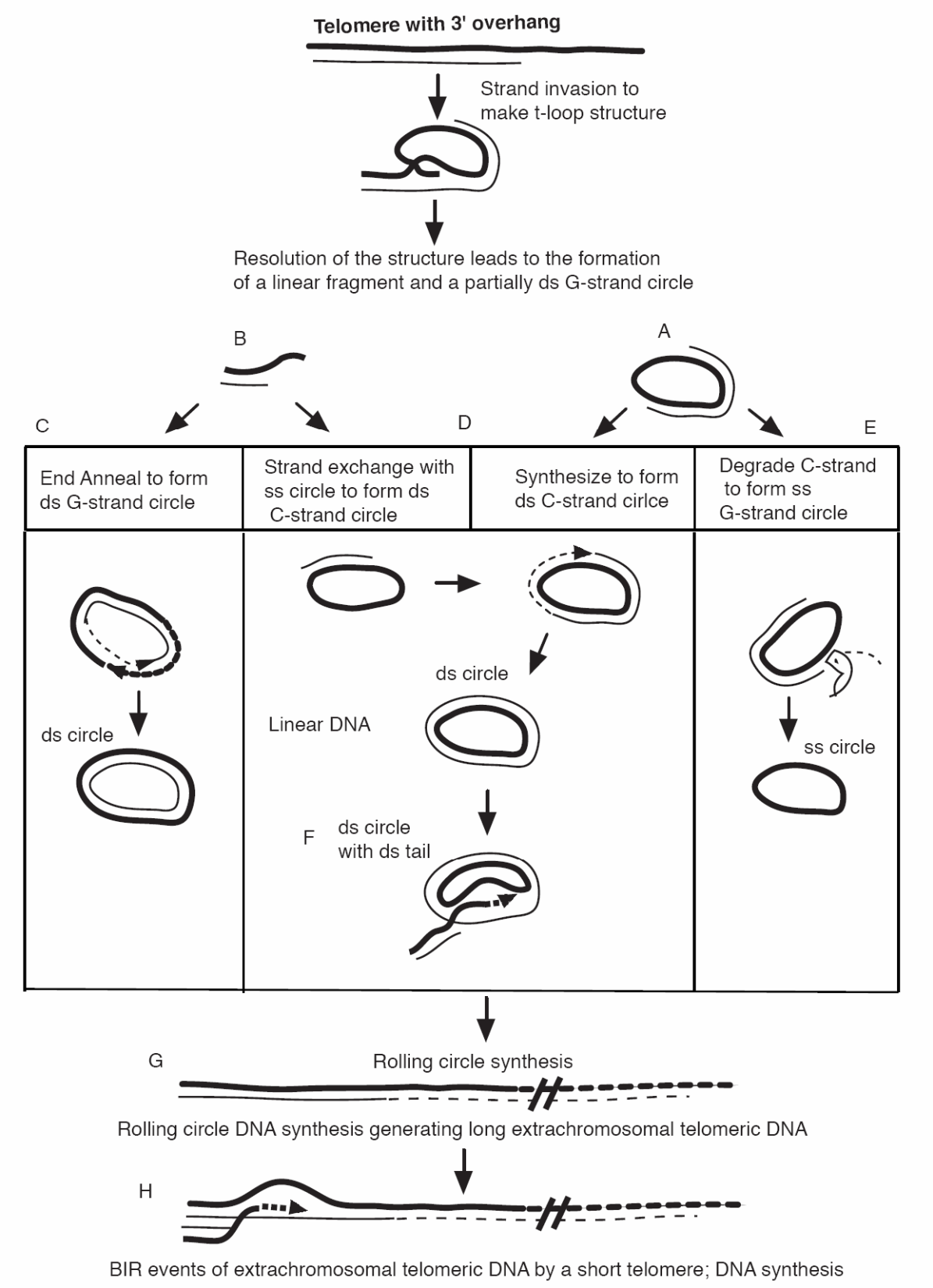
Telomere with 3' overhang
Strand invasion to make t-loop structure
Resolution of the structure leads to the formation of a linear fragment and a partially ds G-strand circle
B
A
C
D
E
End Anneal to form ds G-strand circle
Strand exchange with ss circle to form ds C-strand circle
Synthesize to form ds C-strand cirlce
Degrade C-strand to form ss G-strand circle
ds circle
Linear DNA
ds circle
ss circle
F
ds circle with ds tail
G
Rolling circle synthesis
Rolling circle DNA synthesis generating long extrachromosomal telomeric DNA
H
BIR events of extrachromosomal telomeric DNA by a short telomere; DNA synthesis

Figure 3-10

CHAPTER 4

THE ROLE OF NHEJ-COMPONENTS IN TELOMERE METABOLISM IN *KLUYVEROMYCES LACTIS*[1]

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[1]Shilpa Iyer[*], Sidney Carter[*], Michael J. McEachern and Stefan U. Åström. 2006. Molecular and Cellular Biology. Submitted. [*]-Equal contribution.

ABSTRACT

We investigated the role of the nonhomologous end joining (NHEJ) pathway in telomere dynamics in *Kluyveromyces lactis*. A specific allele of the *TER1* gene (*ter1-4LBsr*), encoding the RNA component of telomerase resulted in telomere-telomere fusions. Examination of the fusions revealed a variable number of mutant telomeric repeats, but only few wild type repeats. Both DNA Ligase 4 and Yku80 were necessary for fusions to arise, consistent with the NHEJ-pathway mediating the fusion events. However, strains lacking Nej1, a protein necessary for NHEJ at internal double-stranded breaks (DSBs), formed fusions with similar kinetics compared to the wild type. Hence, NHEJ-mediated telomere fusions had different molecular requirements than NHEJ at other parts of the genome. Strains lacking the *rad50* and *mre11* genes, exhibited stable short telomeres, were deficient for NHEJ. Introducing the *ter1-4LBsr* allele into these strains failed to elongate telomeres, consistent with their observed role in telomerase recruitment in *S. cerevisiae*. To explore the role of the NHEJ components in the maintenance of wild type telomeres, a sub-telomere gene conversion assay was performed. The results showed a substantial increase in recombination rates near telomeres in *rad50* Δ and *mre11*Δ strains, similar to other short telomere mutants in *K. lactis*. Strains lacking Ku80 displayed a modestly elevated gene conversion rate despite having normal length telomeres. We also observed extended single-stranded 3'overhangs in the *ku80* Δ strain, consistent with a telomere-capping defect. We conclude that NHEJ proteins have multiple roles at *K. lactis* telomeres, mediating fusion of long mutant telomeres and ensuring end protection of wild type telomeres.

INTRODUCTION

Telomeres are specialized protein-DNA complexes at the ends of linear chromosomes. Telomeric DNA in most eukaryotes consists of tandem repeats of short G-rich sequences. In *Saccharomyces cerevisiae* and *Kluyveromyces lactis* telomeric repeats are bound by the Rap1 protein (Repressor activator protein 1) (13, 38), a sequence specific DNA-binding protein containing two Myb-domains. Myb-domain proteins binds to telomeres in other organisms as well, for example Taz1 in fission yeast (14), and Trf1/Trf2 in mammals (10).

Telomeres are synthesized by a specialized enzyme called telomerase (11). In both *S. cerevisiae* and *K. lactis*, the catalytic core of this enzyme contains Est2 a reverse transcriptase and an internal RNA component (*TLC1* in *S. cerevisiae* and *TER1* in *K. lactis*) used as template during DNA synthesis. Complete loss of telomerase leads to the gradual shortening of telomeres and eventual replicative senescence, due to the inability of the normal replicating machinery to fully replicate linear DNA ends (42).

Natural chromosome ends are protected from fusions with other DNA ends indicating that telomeres have properties that make them different from DNA double strand breaks (DSBs) that arise elsewhere in the genome. This protective feature of telomeres, first observed by McClintock (45) and Muller (53), is often referred to as the telomere cap-function. One result of a compromised telomere cap is that chromosome termini are considered to be *bona fide* DSBs and that cells will attempt to repair these breaks. One pathway for DSB-repair is the homologous recombination (HR) pathway, dependent on the *RAD52* group of genes (68). The hallmark of the HR pathway is that a sister chromatid or chromosome provides a homologous template during a mostly error-free repair process.

In contrast, the nonhomologous end joining (NHEJ)-pathway for DSB-repair (15)

requires little or no homology and simply fuses two free DNA-ends, often generating small deletions and insertions. Normally, these ends are the result of a DSB that arise in internal positions of chromosomes. The *K*u70/Ku80 heterodimer (50) is required for NHEJ and is thought to protect and align the DNA ends for subsequent end-processing and ligation (19). The DNA ligase, Lig4 (Dnl4 or Ligase IV) (65, 69, 77) and Lif1 (Ligase four interacting factor 1) (30) provides the ligase activity during NHEJ. Lif1 is the yeast functional homologue of mammalian Xrcc4 and is required for the stability and full activity of Lig4 (70). In addition, the MRX complex (Mre11, Rad50, and Xrs2), is also required for efficient NHEJ in *S .cerevisiae* (8, 52). Mre11 is an endo/exo nuclease, but mutant forms of Mre11 lacking detectable nuclease activity still supports NHEJ (40). Cell-type regulates NHEJ in *S. cerevisiae*, such that haploid *MAT***a** or *MAT*alpha strains perform NHEJ efficiently, but diploid *MAT***a**/*MAT* alpha strains perform NHEJ inefficiently (1, 39). The *NEJ1* gene is transcribed in haploid but not diploid cells, encodes a protein that is essential for NHEJ and that interacts with Lif1 (24, 34, 57, 74).

It is widely accepted that telomeres with a compromised cap can engage in both homologous recombination (HR) and nonhomologous end joining (NHEJ). HR-repair can result in telomerase independent telomere elongation or telomere shortening. In contrast, NHEJ-repair of telomeres results in telomere-telomere fusions (T-Tfs). An illustration of this is telomerase deficient fission yeast in which the majority of cells die. In most surviving cells chromosomes will circularize, as a result of T-Tfs and a smaller fraction of survivors appear to maintain their telomeres by recombination (54). However, in *S. cerevisiae*, both telomerase and the Tel1 protein kinase, a member of the ATM family, protect telomeres from fusions (12, 49).

Other mutations, not directly involved in telomerase function, can also compromise the telomere cap. For example, overexpression of a dominant-negative version of the human

telomere protein hTrf2 resulted in telomere fusions in which telomere sequences were still present (75). Fission yeast Taz1 (20) and *S. cerevisiae* Rap1 (59) are also required to avoid telomere fusions, indicating that these Myb domain proteins are part of the DNA-protein complex constituting the cap. In *K. lactis*, Ttfs are observed in strains with mutations in the template region of the telomerase RNA, which are predicated to abolish or decrease Rap1 binding (48). Telomere-telomere fusions appear to arise by an NHEJ-dependent mechanism since these fusions requires Lig4, a NHEJ-specific ligase, in both yeasts and mammals (21, 41, 49, 66). The role of individual NHEJ components in generating telomere fusions is not clear, however. *S. cerevisiae* Ku has been reported to be both required (59) and dispensable (49) for TTfs. Paradoxically, knock-down alterations of mammalian Ku86 expression promote chromosome fusions involving telomeric sequences (31, 64) suggesting that Ku acts to prevent telomere fusions. In addition, *S. cerevisiae* Nej1 has been reported to prevent telomere fusions, despite being required for promoting NHEJ (41).

In this study, we have explored the role of NHEJ proteins in telomere metabolism in *K. lactis*,investigating both telomere fusions and levels of subtelomeric gene conversion. We found that Ku80 and Lig4 were required for formation of telomere fusions and that subtelomeric gene conversion rates were dramatically increased in strains lacking Mre11 or Rad50. In addition, Nej1 was not required for telomere fusions, but was required for NHEJ in other assays.

RESULTS

Telomere length in strain lacking NHEJ-components

To determine if NHEJ-proteins were required for maintaining normal telomere length in *K. lactis*, we determined telomere lengths in wild type and NHEJ mutant strains using Southern blots (Figure 4-1). This analysis revealed that *lig4*, *yku80* and *nej1* mutant strains had normal telomere lengths, but telomeres were shorter in *rad50* and *mre11* mutants. The shortened telomeres in strains lacking the MRX-complex were consistent with previous results obtained in *S. cerevisiae* (71). The observation that a *yku80* mutant strains had normal length telomeres was surprising since the corresponding mutation leads to telomere shortening in *S. cerevisiae*.

Next we explored if we could observe a telomere length defect in the absence of NHEJ proteins in a different mutant background. It has previously been shown that strains lacking Sir4 in *K. lactis* have longer telomeres than wild type controls (2). We compared the telomere length of a *sir4* single and a *sir4 yku80* double mutant strain and found that the double mutant had telomeres longer than the wild type strain, but shorter than the *sir4* single mutant strain (Figure 4-1B). In contrast, telomere length in the *nej1 sir4* and *lig4 sir4* double mutant strains were similar to the *sir4* single mutant strain, confirming that NHEJ function was not required for maintaining normal telomere length (Figure 4-1B). The telomere length in the *mre11 sir4* double mutant strain was shorter than those of wild-type cells, but longer than those of the *mre11* single mutant, indicating that Mre11 and Sir4 affected telomere length homeostasis by different mechanisms.

Single-stranded 3' overhangs were formed in the *Ku80* mutant.

Since the deletion of *ku80* did not alter the telomere length in an otherwise wild type background, we further investigated its role in protecting the chromosome ends. Work in *S. cerevisiae* has demonstrated that Ku plays a role in capping the telomeres. Disruption of the Ku subunits resulted in telomere shortening, while at the same time the single-stranded overhangs became longer (4, 5, 9, 27, 69). It has also been seen that one of the consequences of telomere uncapping is the generation of extensive overhangs, due to the degradation of 5' end (25). Therefore we analyzed *ku80*, *rad50*, *mre-11* mutants using non-denaturing in-gel hybridizations (16). Genomic DNA from each of these strains was digested separately with *Pst*I, *Msp*I and *Pvu*II, to distinguish slight perturbations in telomere length, and electrophoresed on two 0.7% agarose gels (Figure 4-4A). Subsequent efforts focused on detecting the presence of 3'single-stranded DNA. The partially dried gel was sliced into two equal halves, and one half was hybridized to the 5'cytosine rich strand (C-strand) and the second half to the telomere oligonucleotide probe. As a positive control a second gel was electrophoresed in an identical manner, denatured and hybridized to the 3'guanine rich strand (G-strand) telomere oligonucleotide probe. A strong signal with the C-strand telomere probe was visible only in the *ku80* strain, while hardly any signal was detected in the wild type or the *mre-11* and *rad 50* mutant strains, consistent with the presence of extensive 3' single-stranded DNA in the *ku80* mutant. The denatured gel showed telomeric signals in all the lanes. Hence the absence of signal in the non-denaturing gel was not due to the absence of DNA. Although, these results are consistent with the *ku80* mutant having a capping defect resulting in the presence of 3' overhangs,the precise length of these overhangs and whether they were cell cycle dependent remains unclear.

Increased subtelomeric recombination occurs in *rad50, mre11, lig4* and *ku80* mutants.

Both shorter telomere length and elongated 3' telomere overhangs have been associated with increased recombination in and near telomeres (33, 47). We, therefore, hypothesized that *ku80, mre11, and rad50* cells would exhibit elevated levels of subtelomeric recombination. To test this idea, we performed assays on *ku80*, *mre-11*, *rad50*, *lig4* and wild type cells to measure the rates of sub-telomeric gene conversion (47). Briefly, telomere DNA, tagged with a *URA3* marker gene at the subtelomere, was transformed into the above-mentioned strains. Twelve individual transformants from each strain were shown to contain a single copy of the *URA3*-tagged telomere using Southern blot analysis, replacing the native telomere (see Material and Methods). As described previously, the frequency of *URA3* loss due to gene conversion events was then measured by plating serial dilutions of the cells on medium containing 5-FOA (47). Relative to the wild type control strain SAY45, we observed dramatic increases in *URA3* loss (Table 1). The ~250-1000 fold increased gene conversion rates in *rad50* and *mre11* indicate that these mutants were particularly compromised in their ability to protect telomere and sub-telomeric regions against recombination. These results was consistent with past work which showed that *K. lactis* mutants with telomeres stably shortened to a length much below normal size were subjected to highly elevated subtelomeric BIR events (47, 55). An interesting observation is a modest increase in sub-telomeric recombination in the absence of Lig4. This is the first indication that Lig4 might be involved in telomere end- protection. Our results indicate that Ku80 and Lig4 mutants have a telomere-capping defect, regardless of normal telomere length.

Telomere-telomere fusions were produced by an atypical NHEJ mechanism

Mutations in the template region of telomerase RNA, encoded by the *TER1* gene in *K. lactis*, lead to the introduction of the corresponding changes in the newly synthesized telomere repeats. Specific mutations in the *TER1* gene, predicted to abolish Rap1 binding to the newly synthesized repeats, can lead to the formation of telomere-telomere fusions (48). We used strains containing a combination of one of these mutations called *ter1-4L*:Bsr (abbreviated *ter1-4L*) and mutations in NHEJ-components and asked if telomere fusions were formed. A useful feature of this particular *TER1* mutant allele is that it introduces a *Bsr*GI restriction site into each newly made telomeric repeat DNA. The NHEJ-components were inactivated by null mutations in the corresponding genes (*lig4*, *yku80*, *nej1*, *rad50*, *mre11*). These mutations were previously shown to confer a defect in NHEJ in a plasmid re-joining assay (37).

Diploids heterozygous for both the *ter1-4L* and NHEJ mutations were subjected to tetrad analysis and the progeny from these crosses were analyzed for marker segregation. The haploid strains had normal telomeres after spore germination because the *TER1* locus was duplicated such that both the wild type *TER1* gene and the *ter1-4L* gene were present with an intervening *URA3* marker gene with the wild type gene being epistatic to the mutant allele (48). After a pop-out procedure on agar plates containing 5-FOA, segregants in which the mutant allele was maintained were recovered. From each cross we also recovered a *ter1-4L* single mutant strain as a control. The *ter1-4L* single mutant strains showed the expected pattern of immediate severe telomere elongation and degradation (Figure 4-2, and data not shown), but after prolonged passaging sharp discrete bands appeared. These bands were previously shown to be resistant to *Bal*31 exonuclease digestion (48) and are indicative of telomere fusions. Two independent isolates of strains lacking Lig4 or Yku80 showed no signs of telomere fusions even after 25

streaks, demonstrating that these proteins were required for telomere-telomere fusions. In contrast, strains lacking Nej1 formed telomere fusions and the formation of these fusions followed the same rate as formation of fusions in a wild type strain (data not shown). We concluded that telomere-telomere fusions in *K. lactis* appeared to arise by an atypical NHEJ mechanism, which required Lig4 and Yku80, but did not require Nej1.

Combining the *ter1-4L* mutation with *rad50* or *mre11* mutations revealed a surprising result with respect to the initial telomere elongation phenotype. The *ter1-4L rad50* and the *ter1-4L mre11* double mutant strains did not show a loss of telomere length regulation and the telomeres remained stably short even after 25 streaks (Figure 4-2). Digestion with the *Bsr*GI restriction enzyme showed, however, that a few mutant repeats had been incorporated (Figure 4-2, and data not shown). The number of repeats incorporated was probably as few as one or two, as estimated from the size difference on DNA blots (Figure 4-2).

Molecular characterization of telomere fusions

The fusions between telomeres were further characterized using a PCR strategy similar to a previously described strategy in *S .cerevisiae* (49, 59). We took advantage of the known sequence of subtelomeric regions in *K. lactis* (56) and designed four PCR primers that annealed within the subtelomeric regions of *K. lactis* telomeres. We tested the specificity of these primers in different combinations using chromosomal DNA from non fused and post-fusion strains, as templates. One primer pair specifically and consistently amplified DNA from several post-fusion strains (Figure 4-3), resulting in a smearing signal. This primer pair corresponded to a primer complementary to a sequence unique to the left end of chromosome II (P1) and another primer that was complementary to at least nine subtelomeric regions (P2-11) (See materials and

methods for details). PCR with only primer P2-11 failed to generate a product. This result did not rule out fusions between telomeres not involving telomere 2L, since such fusions probably resists PCR amplification being almost perfect palindromes.

The PCR product from two post fusion strains were cloned, and amplified in *E. coli*. Digestion with *Bsr*GI enabled us to make estimations on the number of *Bsr*GI containing telomeric repeats. In addition, DNA sequencing made it possible to count most wild type repeats. The template DNA was from a late streak (25) and an early streak (5), and the two strains were from independent crosses. Given the smeary nature of the signal three or four independent clones were sequenced. In all cases, the anticipated subtelomeric sequences were found, but the palindromic telomere repeats could not be sequenced through the fusion point. The three clones originating from the late streak (Fusion 1-3, Figure 4-3) had very few Bsr*GI* containing repeats, probably only one. The four clones originating from the early streak, however, contained numerous mutant repeats [4-19]. In all of the fusions sequenced, the number of wild type repeats on each end was very limited [0 to 5].

This PCR-based assay for T-Tfs was probably much more sensitive than the DNA-blots used previously. Therefore, we investigated if telomere fusions could be detected in strains with mutations in NHEJ components using the PCR-based assay. The results showed evidence of telomere fusions in *nej1* mutant strains, but not in strains lacking Yku80, Lig4, Mre11, or Rad50 (data not shown). The two assays used for T-Tfs were thus equivalent and generated the same results.

Multiple T-Tf s are highly detrimental to *K. lactis* meiosis.

The *ter1* template mutants that cause fusions between most or all telomeres have been postulated to select for derivatives that have circularized each of their six individual chromosomes (48). Mutant cells with no detectable free telomeres and six telomere fusions displayed only moderately slow mitotic growth. Nevertheless, such cells were expected to incur difficulties while undergoing meiosis. This is because crossovers involving a circular chromosome would dimerize a homologous chromosomal pair and interfere with their disjunction at meiosis I. To test this, we used a *ter1-AccSna* mutant for conducting mating and sporulation analysis. This mutant strain, which contains a single base substitution in both the left half and right half of the Rap1 binding site (Figure 4-5) is slower to undergo fusions than a *ter1-BsrG* mutant strain. However, the T T-fs formed in this strain are more stable, because of the smaller average sizes of the fused telomere sequences.

A *ter1-AccSna* fusion strain showed no defect in the ability to mate with GG1958, a strain with normal telomeres, when compared to an isogenic 7B520 wild type control. However, when diploids constructed by crossing control haploid 7B520 and GG1958 strains and *ter1-AccSna* and GG1958 strains were sporulated, a dramatic difference in successful tetrad production was observed. Microscopic observation showed that tetrads were abundant among cells of the control strain but seemingly absent from the diploids derived from the telomere fusion strain. To further characterize this meiotic defect, random spore analysis was performed on the sporulating cultures of the same diploid strains. Survival of the spore cells, following heat treatment at 55 °C, was consistently reduced by ~100 fold for cells derived from the *ter1-AccSna* diploids relative to the diploid controls. Those cells that did survive exhibited one or more phenotypes (Ade-, Ura-, His-), which suggested that they were derivatives from the same

haploid parental strains that were used to set up the initial cross. We next examined the structure of the telomeres in sixteen of these segregants from the *ter1-AccSna* fusion diploids by Southern hybridizations. The results (Figure 4-5), confirmed that each of the clones contained mixtures of fused and unfused telomeres. The unfused telomeres were either all of normal length or all shorter than normal, consistent with the presence of either wild type *TER1* or *ter1-AccSna* (which produces short telomeres prior to fusion formation). In most cases, the fusion bands were identical in size with those of the *ter1-AccSna* haploid parent, consistent with them being unaltered by meiosis. In at least two instances (Figure 4-5, lanes 14 and 15) novel sharp bands were also visible in *ter1-AccSna* haploids.

DISCUSSION

In this work, we examined the role of several *K. lactis* gene in the regulation of telomere length and the ability of the affected telomeres to protect against homologous recombination and non-homologous end joining. Our data shows that telomere length regulation is governed by a variety of proteins and processes, as numerous gene mutations resulted in altered telomere length. Several of the gene knockouts *ku80*, *mre-11*, *rad50* we examined produced changes in the length of telomeres. The modest telomere elongation observed in a *K. lactis sir4* mutant differs from the result observed in *S. cerevisiae*, where both *sir3* and *sir4* mutations both produce modest telomere shortening (58). However, our results are consistent with the Sir protein complex being present at *K. lactis* telomeres and contributing in a minor way to telomere length regulation. It is also likely that the Sir proteins are responsible for the transcriptional silencing that can occur near *K. lactis* telomeres (58) as is the case in *S. cerevisiae* (28).

In *S. cerevisiae*, unusually short but stable telomere lengths have been seen upon deletion of certain genes in the DNA repair pathways including the Ku, the MRX complex (composed of Mre11, Rad50 and Xrs2) and the Tel1 kinase (8, 9, 36). Genetic analysis has shown that MRX and Tel1 function in the same pathway of telomere maintenance but in a different pathway than Ku (63). Using Southern hybridizations, we show here that in *K. lactis*, deletion of *MRE11* and *RAD50* genes also led to stably shortened telomeres. These data argue that the *K. lactis* MRX complex functions in maintenance of telomere length in much the same way that it does at *S. cerevisiae* telomeres.

We also showed here that both *mre11* and *rad50* mutants of *K. lactis* displayed large increases in the rate of subtelomeric BIR events. Similarly large increases in subtelomeric BIR were previously seen in *K. lactis* telomerase RNA gene (*TER1*) mutants with stably shortened telomeres (47). The increased subtelomeric BIR rates seen in *mre11* and *rad50* mutants may therefore be an indirect consequence of shorter telomere length rather than an additional separate defect in telomere capping. A previous report found that *S. cerevisiae mre11* and *rad50* mutants did not display a large increase in the rate of telomeric recombination despite having very short telomeres (17). However, this study employed a telomere capture assay that required recombination with a telomeric repeat tract that was only ~80 bp in length. Subsequent work has shown that recombination involving homologous sequences of <100 bp can require *RAD50* for function (32). We hypothesize that subtelomeric BIR events in *K. lactis mre11* and *rad50* mutants are Rad51-dependent. A Rad51 filament that formed on a single stranded 3' telomeric end could strand invade another telomere with the invasion extending into subtelomeric sequence. Certain outcomes of this strand invasion complex could result in loss of the *URA3* and replacement of the original telomeric repeats with sequence copied from the donor molecule. As

the subtelomeric sequence shared between at least 11 of 12 *K. lactis* telomeres extends for well over 1 kb, there would be ample homology available for Rad51-dependent recombination to occur, even if the adjoining telomeric repeat tract was very short.

The Ku70/Ku80 heterodimer has been shown to be involved in both telomere length regulation and telomere capping in *S. cerevisiae* (4, 5, 8, 15, 18, 26, 27, 31, 67). Loss of either Ku70 or Ku80 caused a large decrease in telomere length and an increase in the length of 3' overhangs present at telomeres (4). These two roles are genetically separable (5). Ku's effect on telomere length appears to result from its ability to bind directly to telomerase RNA and help recruit telomerase to telomeres. The *S. cerevisiae* Ku makes specific contacts with a particular stem loop region of the telomerase RNA (51, 60, 67).

An interesting finding of this study is that loss of the *K. lactis YKU80* gene does not appreciably alter telomere length. A possible explanation for this result is that the binding site for Ku at the stem loop region is absent in the telomerase RNA of *K. lactis* (72). Therefore, the *K. lactis* Ku protein may not have a major role in recruitment of telomerase to telomeres. However, an *yku80sir4* double mutant did exhibit slightly shorter telomeres than a *sir4* single mutant, consistent with Ku having a minor role in telomere length regulation. Although a Ku deficiency leads to telomere shortening in many organisms studied to date (23), there are exceptions to this rule. For example loss of Ku in *Arabidopsis*, results in considerable telomerase dependent telomere elongation (62).Several studies reported a role for Ku in telomere capping [Reviewed in (23)]. Ku deficiency has been reported to trigger an increased recombination (3, 17, 51, 61) and increased degradation of 5' strand of telomeric DNA (27, 44, 61). Our results are consistent with these observations as we show that loss of *yku80* results in increased single stranded 3' overhangs and an increase in subtelomeric recombination.

Another interesting observation in our study was the increase in the levels of subtelomeric recombination in the *lig4* strain. DNA ligase IV (Lig4), is an essential part of the NHEJ pathway along with Ku80 in DSB repair, but not role has yet been assigned to Lig4 in telomere metabolism.. Therefore, the substantial increase in recombination near telomeres in a *K. lactis* lig4 mutant is the first evidence of Lig4 contributing to chromosome end protection at the telomeres. Alternatively, HR and NHEJ compete for repairing DSBs in subtelomeric regions and absence of Lig4 shuttles more repair events into the HR pathway. In support of this idea, we have observed competition between HR and NHEJ for an ectopic DSB induced ~ 25kb from a telomere in K. lactis (Paula Martinez and S.U.Å., unpublished observation). It will be important to ascertain whether *K. lactis lig4* mutants, like *yku80* also exhibit increased 3' overhang size. It will also be worthwhile to conduct further experiments to ascertain if Lig 4 plays a role in telomere maintenance in the absence of telomerase, a major pathway in length regulation.

In addition to studying the roe of NHEJ components on telomere homeostasis, we also studied their requirement for telomere fusion (Ttfs) formation in *K. lactis*. On rare occasions, chromosome circularization has been observed in linear genomes of yeasts and mammals, as a result of a compromised telomere cap (33, 54). Several lines of evidence have pointed out that the genetic requirements for the formation of Ttfs can be different between species. Work in *S. pombe* has shown that in the absence of the telomerase component, chromosomes circularized (54). However, these fusions formed independently of Ku80 and Lig IV(22). Other types of telomere fusions occurred in mutants lacking Taz1 (20). These mutants exhibited long telomeres and the fusion in these strains were dependent on Ku80 and Ligase IV.

Previous work in *K. lactis* has shown that mutants containing different allelic mutations in the RNA component of telomerase altered the binding site of the protein Rap1, eventually

resulted in telomere fusions and apparent circularization of all six chromosomes (48). These fusions seem to retain a sizeable number of telomeric repeats. In this study, we showed that the fusions formed in a *ter1-4L*Bsr strain contained a variable number of mutant telomeric repeats, but only few a wild type repeats. In general, a late streak of the *ter1-4L*Bsr mutant strain appeared to contain fusions with fewer mutant telomeric repeats than fusions obtained from an early streak. Shorter fusions are estimated to be more stable and this difference may indicate a shift of the fused telomeres to a more stable state. However, as observed with DNA blots, the size of telomere fusions in the *ter1-4L*Bsr mutant strains appeared to vary between individual isolates. This variation could also be responsible for the observed difference in mutant telomere repeat number between the assayed early and late streaks. In all T-Tfs sequenced very few wild type repeats were present indicating that very few Rap1 molecules were bound to the telomeres prior to fusions. This presumably led to uncapping and a possibility for the NHEJ pathway to fuse the telomeres. We found that Ku80 and Lig4 were required for formation of telomere fusions providing the first evidence that fusions *ter1-4L* mutant was dependent on the NHEJ pathway. Secondly, deleting *RAD50* and *MRE11*, also required for NHEJ at internal DSBs, led to very short telomeres and lack of any T-Tfs. The simplest explanation for this result was that Rad50 and Mre11 were required for both telomere length homeostasis and for the formation of T-Tfs. Another explanation for this result could be that a certain number of telomeric repeats were required to form a T-Tf.

The key finding from this body of work was that strains lacking Nej1, a protein necessary for NHEJ at internal DSBs, formed fusions with similar rates compared to the wild type. One possibility was that the function of Nej1 was replaced by another protein during the formation of T-Tfs. It was also possible that the nature of NHEJ at telomere termini differs from NHEJ at

internal chromosome positions in a manner as to render Nej1 function dispensable. Given that the role of Nej1 during NHEJ is unknown, it is difficult to speculate about the nature of such a function. In any event, NHEJ-mediated telomere fusions had different molecular requirements than NHEJ at other parts of the genome.

It has been observed previously that *S. pombe* strains containing T-tfs, displayed severe defects in their ability to undergo meiosis, a problem inherent to circular chromosomes. Our results have shown that *ter1- AccSna* cells with Ttfs also have a severe problems going through meiosis. A possible explanation for the rare spores that did go through meiosis was that even number of cross over events took place between each homologous pair. This would prevent formation of dicentric chromosomes and enable chromosome disjunctions with fused telomeres, thus allowing the cells to proceed through meiosis. An alternate possibility could be that passage through meiosis requires the presence of telomeric sequences, since fusions in *ter1- AccSna* strains retain telomere sequences. Consistent with this idea, in *K. lactis,* it has been found that mutant telomere repeats interfere with meiosis (43).

MATERIALS AND METHODS

Yeast strains

Strains lacking *LIG4* (AKY124)*, NEJ1* (SAY572), *KU80* (SAY573)*, MRE11* (SAY579) and *RAD50* (SAY557) were generated as previously described (34). The double mutant strains *sir4 lig4* (SAY703), *sir4 nej1* (SAY701), *sir4 ku80* (AKY116) and *sir4 mre11* (SAY704) were generated by crossing a *sir4* strain (SAY100) with the respective non-homologous end-joining mutant strain followed by tetrad analysis. For telomere-telomere fusion assays, strains containing

a duplicated *TER1* locus containing both a wild-type and the mutant *ter1-4L-BsrG1* allele were crossed to strains carrying null mutations in *LIG4, NEJ1, KU80, MRE11* and *RAD50*. Tetrad analysis resulted in the isolation of *TER1::URA3-ter1-4L-BsrG1 ku80* (SAY600), *TER1::URA3-ter1-4L-BsrG1 mre11* (SAY579), *TER1::URA3-ter1-4L-BsrG1 rad50* (SAY604), *TER1::URA3-ter1-4L-BsrG1 nej1* (SAY562), and *TER1::URA3-ter1-4L-BsrG1 lig4* (SAY563) double mutant strains. The *TER1::URA3-ter1-4LbsrG1 nej1 lig4* triple mutant strain (SAY564) was obtained through the same procedure following a cross between SAY552 and a *nej1 lig4* double mutant strain (SAY545). Each strain was subsequently plated on 5-FOA medium (7), resulting in the replacement of the wild-type *TER1* gene with the mutant *ter1-4L-BsrG1* allele. The resulting strains were maintained by serial re-streaks on rich medium (YEPD) for 48 to72h at 30°C.

Random spore analysis

Analysis was performed on the spores generated by mating *ter1-AccSna* strain (*ura3, his2, trp1)*, with GG1958 (*ade2*). Diploids cells were transformed to sporulation media for four days. Spores isolated from these diploid cells, were incubated in 200 ul of Zymolyase (concentration of 0.17 mg/ml) in1M Sorbitol at 37° C for 10 minutes to digest the ascus sac. Subsequently, they were subjected to heat treatment at 54°C for a further 10 minutes to kill all the vegetative cells. The resulting spores were serially diluted in TE (10mM Tris and 1mM EDTA) and plated on YPD plates.

Hybridizations

DNA blots were hybridized with the previously described wild-type *K. lactis* telomeric probe (Klac1-25) at 50°C following a standard protocol(46). In-gel hybridization experiments

were described as previously (16), using telomere oligonucleotide as a probe (Klac25-1). Approximately 3 ug of digested genomic DNA was electrophoresed through a 0.7 % agarose gel and then the contents were analyzed as described previously (73).

Subtelomere gene conversion assay

The gene conversion assay was performed according to the protocols described previously (47). In brief, the native telomere in *rad50,mre-11,ku80, lig4,* WT SAY45, strains was replaced by transformation with an ~2.0-kb *Eco*RI and *Sac*II "STU" (subtelomere,telomere,*URA3*) fragment containing the URA3 gene from *S. cerevisiae*, was inserted close to the junction of the cloned telomere and subtelomere fragment of *K. lactis*. Twelve individual transformants from each of the strains, were digested with EcoRI, and hybridized to a *URA3-Rad52*gene fragment probe and a telomere probe to test for the presence of a new band containing the single *URA3* gene. The ratio of the individual signal was quantitated against each *RAD52* band indicating a single copy of the *URA3* gene marker. Serially diluted cells of the clones containing the "STU" fragment were plated on plates containing synthetic complete (SC) lacking uracil, SC plus 5-FOA, and YPD. Measurement of the loss of the URA3 gene was performed by counting colonies grown on 5-FOA with respect to the total number of colonies grown on YPD and SC.

Analysis of telomere-telomere fusions

The strains used for analysis of telomere-telomere fusion were *ter1-4L*Bsr strains SAY605 and SAY561 following twenty-five and five serial re-streaks, respectively. Genomic DNA was prepared using MasterPure Yeast DNA Purification Kit (EPICENTRE, Madison, WI).

One primer (P1 5'-CAGGGCGGGTAACATGAC-3') contained sequence unique to *K. lactis* chromosome 2L and corresponded to 268-285 nucleotides upstream of the telomeric repeat sequence start (56). The second primer (P2-11 5' GAAAGAGGAAATCCGTTTCG-3') contained sequence common to subtelomeric region of at least nine chromosome ends other than 2L. Potential annealing sites for this primer ranged between 167-254 nucleotides from the telomeric repeat sequence start, depending upon the chromosome end (56). PCR reactions (50µl) contained 0.2ng genomic DNA, 1X PCR reaction buffer (10mM Tris-HCl, 1.5mM MgCl2, 50mM KCl), dNTP 0.1mM each, primers 0.5µM each and 1U Taq DNA polymerase. Reaction conditions were as follow: 95°C 1min; 35 cycles of 95°C 30s, 55°C 30s, 72°C 2min; followed by 72°C 10min. The products were run on a 1% agarose gel and visualized by ethidium bromide staining. Portions of the resulting DNA smear were cut from the gel and the DNA products were recovered. Isolated products were then cloned into the Promega pGEM T-Easy vector using the suggested protocol and transformed into *Escherichia coli* strain DH5α cells for blue-white screening. Approximately 1µg of plasmid DNA was digested with *Eco*R1 or doubly digested with *Eco*R1 and *Bsr*G1. The mixtures were run on a 2% agarose gel and visualized by ethidium bromide staining to determine fragment sizes. Clones were submitted for sequencing by Macrogen (South Korea).

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REFERENCES

1. **Astrom, S. U., S. M. Okamura, and J. Rine.** 1999. Yeast cell-type regulation of DNA repair. Nature **397:**310.

2. **Astrom, S. U., and J. Rine.** 1998. Theme and variation among silencing proteins in Saccharomyces cerevisiae and Kluyveromyces lactis. Genetics **148:**1021-9.

3. **Baumann, P., E. Podell, and T. R. Cech.** 2002. Human pot1 (protection of telomeres) protein: cytolocalization, gene structure, and alternative splicing. Mol Cell Biol **22:**8079-87.

4. **Bertuch, A. A., and V. Lundblad.** 2003. The Ku heterodimer performs separable activities at double-strand breaks and chromosome termini. Mol Cell Biol **23:**8202-15.

5. **Bertuch, A. A., and V. Lundblad.** 2003. Which end: dissecting Ku's function at telomeres and double-strand breaks. Genes Dev **17:**2347-50.

6. **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. Science **279:**349-52.

7. **Boeke, J. D., J. Trueheart, G. Natsoulis, and G. R. Fink.** 1987. 5-Fluoroorotic acid as a selective agent in yeast molecular genetics. Methods Enzymol **154:**164-75.

8. **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:**1819-28.

9. **Boulton, S. J., and S. P. Jackson.** 1996. Identification of a Saccharomyces cerevisiae Ku80 homologue: roles in DNA double strand break rejoining and in telomeric maintenance. Nucleic Acids Res **24:**4639-48.

10. **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.

11. **Chan, S. R., and E. H. Blackburn.** 2004. Telomeres and telomerase. Philos Trans R Soc Lond B Biol Sci **359:**109-21.

12. **Chan, S. W., and E. H. Blackburn.** 2003. Telomerase and ATM/Tel1p protect telomeres from nonhomologous end joining. Mol Cell **11:**1379-87.

13. **Conrad, M. N., J. H. Wright, A. J. Wolf, and V. A. Zakian.** 1990. RAP1 protein interacts with yeast telomeres in vivo: overproduction alters telomere structure and decreases chromosome stability. Cell **63:**739-50.

14. **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. Nature **385:**744-7.

15. **Daley, J. M., P. L. Palmbos, D. Wu, and T. E. Wilson.** 2005. Nonhomologous end joining in yeast. Annu Rev Genet **39:**431-51.

16. **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:**13902-7.

17. **DuBois, M. L., Z. W. Haimberger, M. W. McIntosh, and D. E. Gottschling.** 2002. A quantitative assay for telomere protection in Saccharomyces cerevisiae. Genetics **161:**995-1013.

18. **Featherstone, C., and S. P. Jackson.** 1999. Ku, a DNA repair protein with multiple cellular functions? Mutat Res **434:**3-15.

19. **Feldmann, E., V. Schmiemann, W. Goedecke, S. Reichenberger, and P. Pfeiffer.** 2000. DNA double-strand break repair in cell-free extracts from Ku80-deficient cells: implications for Ku serving as an alignment factor in non-homologous DNA end joining. Nucleic Acids Res **28:**2585-96.

20. **Ferreira, M. G., and J. P. Cooper.** 2001. The fission yeast Taz1 protein protects chromosomes from Ku-dependent end-to-end fusions. Mol Cell **7:**55-63.

21. **Ferreira, M. G., and J. P. Cooper.** 2004. Two modes of DNA double-strand break repair are reciprocally regulated through the fission yeast cell cycle. Genes Dev **18:**2249-54.

22. **Ferreira, M. G., K. M. Miller, and J. P. Cooper.** 2004. Indecent exposure: when telomeres become uncapped. Mol Cell **13:**7-18.

23. **Fisher, T. S., and V. A. Zakian.** 2005. Ku: a multifunctional protein involved in telomere maintenance. DNA Repair (Amst) **4:**1215-26.

24. **Frank-Vaillant, M., and S. Marcand.** 2001. NHEJ regulation by mating type is exercised through a novel protein, Lif2p, essential to the ligase IV pathway. Genes Dev **15:**3005-12.

25. **Garvik, B., M. Carson, and L. Hartwell.** 1995. Single-stranded DNA arising at telomeres in cdc13 mutants may constitute a specific signal for the RAD9 checkpoint. Mol Cell Biol **15:**6128-38.

26. **Grandin, N., C. Damon, and M. Charbonneau.** 2000. Cdc13 cooperates with the yeast Ku proteins and Stn1 to regulate telomerase recruitment. Mol Cell Biol **20:**8397-408.

27. **Gravel, S., M. Larrivee, P. Labrecque, and R. J. Wellinger.** 1998. Yeast Ku as a regulator of chromosomal DNA end structure. Science **280:**741-4.

28. **Haber, J. E.** 1999. Sir-Ku-itous routes to make ends meet. Cell **97:**829-32.

29. **Harley, C. B., A. B. Futcher, and C. W. Greider.** 1990. Telomeres shorten during ageing of human fibroblasts. Nature **345:**458-60.

30. **Herrmann, G., T. Lindahl, and P. Schar.** 1998. Saccharomyces cerevisiae LIF1: a function involved in DNA double-strand break repair related to mammalian XRCC4. Embo J **17:**4188-98.

31. **Hsu, H. L., D. Gilley, S. A. Galande, M. P. Hande, B. Allen, S. H. Kim, G. C. Li, J. Campisi, T. Kohwi-Shigematsu, and D. J. Chen.** 2000. Ku acts in a unique way at the mammalian telomere to prevent end joining. Genes Dev **14:**2807-12.

32. **Ira, G., and J. E. Haber.** 2002. Characterization of RAD51-independent break-induced replication that acts preferentially with short homologous sequences. Mol Cell Biol **22:**6384-92.

33. **Iyer, S., A. D. Chadha, and M. J. McEachern.** 2005. A mutation in the STN1 gene triggers an alternative lengthening of telomere-like runaway recombinational telomere elongation and rapid deletion in yeast. Mol Cell Biol **25:**8064-73.

34. **Kegel, A., J. O. Sjostrand, and S. U. Astrom.** 2001. Nej1p, a cell type-specific regulator of nonhomologous end joining in yeast. Curr Biol **11:**1611-7.

35. **Kim, N. W., M. A. Piatyszek, K. R. Prowse, C. B. Harley, M. D. West, P. L. Ho, G. M. Coviello, W. E. Wright, S. L. Weinrich, and J. W. Shay.** 1994. Specific association of human telomerase activity with immortal cells and cancer. Science **266:**2011-5.

36. **Kironmai, K. M., and K. Muniyappa.** 1997. Alteration of telomeric sequences and senescence caused by mutations in RAD50 of Saccharomyces cerevisiae. Genes Cells **2:**443-55.

37. **Kooistra, R., P. J. Hooykaas, and H. Y. Steensma.** 2004. Efficient gene targeting in Kluyveromyces lactis. Yeast **21:**781-92.

38. **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.

39. **Lee, S. E., F. Paques, J. Sylvan, and J. E. Haber.** 1999. Role of yeast SIR genes and mating type in directing DNA double-strand breaks to homologous and non-homologous repair paths. Curr Biol **9:**767-70.

40. **Lewis, L. K., F. Storici, S. Van Komen, S. Calero, P. Sung, and M. A. Resnick.** 2004. Role of the nuclease activity of Saccharomyces cerevisiae Mre11 in repair of DNA double-strand breaks in mitotic cells. Genetics **166:**1701-13.

41. **Liti, G., and E. J. Louis.** 2003. NEJ1 prevents NHEJ-dependent telomere fusions in yeast without telomerase. Mol Cell **11:**1373-8.

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

43. **Maddar, H., N. Ratzkovsky, and A. Krauskopf.** 2001. Role for telomere cap structure in meiosis. Mol Biol Cell **12:**3191-203.

44. **Maringele, L., and D. Lydall.** 2002. EXO1-dependent single-stranded DNA at telomeres activates subsets of DNA damage and spindle checkpoint pathways in budding yeast yku70Delta mutants. Genes Dev **16:**1919-33.

45. **McClintock, B.** 1941. The stability of broken ends of chromosomes in zea mays. Genetics **26:**234-282.

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

47. **McEachern, M. J., and S. Iyer.** 2001. Short telomeres in yeast are highly recombinogenic. Mol Cell **7:**695-704.

48. **McEachern, M. J., S. Iyer, T. B. Fulton, and E. H. Blackburn.** 2000. Telomere fusions caused by mutating the terminal region of telomeric DNA. Proc Natl Acad Sci U S A **97:**11409-14.

49. **Mieczkowski, P. A., J. O. Mieczkowska, M. Dominska, and T. D. Petes.** 2003. Genetic regulation of telomere-telomere fusions in the yeast Saccharomyces cerevisae. Proc Natl Acad Sci U S A **100:**10854-9.

50. **Milne, G. T., S. Jin, K. B. Shannon, and D. T. Weaver.** 1996. Mutations in two Ku homologs define a DNA end-joining repair pathway in Saccharomyces cerevisiae. Mol Cell Biol **16:**4189-98.

51. **Miyoshi, T., M. Sadaie, J. Kanoh, and F. Ishikawa.** 2003. Telomeric DNA ends are essential for the localization of Ku at telomeres in fission yeast. J Biol Chem **278:**1924-31.

52. **Moore, J. K., and J. E. Haber.** 1996. Cell cycle and genetic requirements of two pathways of nonhomologous end-joining repair of double-strand breaks in Saccharomyces cerevisiae. Mol Cell Biol **16:**2164-73.

53. **Muller, H. J.** 1938. The remaking of chromosomes. The Collecting Net **8:**182-195.

54. **Nakamura, T. M., J. P. Cooper, and T. R. Cech.** 1998. Two modes of survival of fission yeast without telomerase. Science **282:**493-6.

55. **Natarajan, S., and M. J. McEachern.** 2002. Recombinational telomere elongation promoted by DNA circles. Mol Cell Biol **22:**4512-21.

56. **Nickles, K., and M. J. McEachern.** 2004. Characterization of Kluyveromyces lactis subtelomeric sequences including a distal element with strong purine/pyrimidine strand bias. Yeast **21:**813-30.

57. **Ooi, S. L., D. D. Shoemaker, and J. D. Boeke.** 2001. A DNA microarray-based genetic screen for nonhomologous end-joining mutants in Saccharomyces cerevisiae. Science **294:**2552-6.

58. **Palladino, F., T. Laroche, E. Gilson, A. Axelrod, L. Pillus, and S. M. Gasser.** 1993. SIR3 and SIR4 proteins are required for the positioning and integrity of yeast telomeres. Cell **75:**543-55.

59. **Pardo, B., and S. Marcand.** 2005. Rap1 prevents telomere fusions by nonhomologous end joining. Embo J **24:**3117-27.

60. **Peterson, S. E., A. E. Stellwagen, S. J. Diede, M. S. Singer, Z. W. Haimberger, C. O. Johnson, M. Tzoneva, and D. E. Gottschling.** 2001. The function of a stem-loop in telomerase RNA is linked to the DNA repair protein Ku. Nat Genet **27:**64-7.

61. **Polotnianka, R. M., J. Li, and A. J. Lustig.** 1998. The yeast Ku heterodimer is essential for protection of the telomere against nucleolytic and recombinational activities. Curr Biol **8:**831-4.

62. **Riha, K., and D. E. Shippen.** 2003. Ku is required for telomeric C-rich strand maintenance but not for end-to-end chromosome fusions in Arabidopsis. Proc Natl Acad Sci U S A **100:**611-5.

63. **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:**475-9.

64. **Samper, E., F. A. Goytisolo, P. Slijepcevic, P. P. van Buul, and M. A. Blasco.** 2000. Mammalian Ku86 protein prevents telomeric fusions independently of the length of TTAGGG repeats and the G-strand overhang. EMBO Rep **1:**244-52.

65. **Schar, P., G. Herrmann, G. Daly, and T. Lindahl.** 1997. A newly identified DNA ligase of Saccharomyces cerevisiae involved in RAD52-independent repair of DNA double-strand breaks. Genes Dev **11:**1912-24.

66. **Smogorzewska, A., J. Karlseder, H. Holtgreve-Grez, A. Jauch, and T. de Lange.** 2002. DNA ligase IV-dependent NHEJ of deprotected mammalian telomeres in G1 and G2. Curr Biol **12:**1635-44.

67. **Stellwagen, A. E., Z. W. Haimberger, J. R. Veatch, and D. E. Gottschling.** 2003. Ku interacts with telomerase RNA to promote telomere addition at native and broken chromosome ends. Genes Dev **17:**2384-95.

68. **Symington, L. S.** 2002. Role of RAD52 epistasis group genes in homologous recombination and double-strand break repair. Microbiol Mol Biol Rev **66:**630-70, table of contents.

69. **Teo, S. H., and S. P. Jackson.** 1997. Identification of Saccharomyces cerevisiae DNA ligase IV: involvement in DNA double-strand break repair. Embo J **16:**4788-95.

70. **Teo, S. H., and S. P. Jackson.** 2000. Lif1p targets the DNA ligase Lig4p to sites of DNA double-strand breaks. Curr Biol **10:**165-8.

71. **Tsukamoto, Y., A. K. Taggart, and V. A. Zakian.** 2001. The role of the Mre11-Rad50-Xrs2 complex in telomerase- mediated lengthening of Saccharomyces cerevisiae telomeres. Curr Biol **11:**1328-35.

72. **Tzfati, Y., Z. Knight, J. Roy, and E. H. Blackburn.** 2003. A novel pseudoknot element is essential for the action of a yeast telomerase. Genes Dev **17:**1779-88.

73. **Underwood, D. H., C. Carroll, and M. J. McEachern.** 2004. Genetic dissection of the Kluyveromyces lactis telomere and evidence for telomere capping defects in TER1 mutants with long telomeres. Eukaryot Cell **3:**369-84.

74. **Valencia, M., M. Bentele, M. B. Vaze, G. Herrmann, E. Kraus, S. E. Lee, P. Schar, and J. E. Haber.** 2001. NEJ1 controls non-homologous end joining in Saccharomyces cerevisiae. Nature **414:**666-9.

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

76. **Vaziri, H., and S. Benchimol.** 1998. Reconstitution of telomerase activity in normal human cells leads to elongation of telomeres and extended replicative life span. Curr Biol **8:**279-82.

77. **Wilson, T. E., U. Grawunder, and M. R. Lieber.** 1997. Yeast DNA ligase IV mediates non-homologous DNA end joining. Nature **388:**495-8.

TABLES

Table 4-1. Elevated levels of recombination near telomeres in *rad50, mre11, ku80, lig4* mutant strains.

The gene conversion rates of the mutants are based on measurement of several (~3-4) independent transformants of the individual mutant strains, containing the *URA3* gene inserted near one telomere (see Material and Methods). For each strain the number of samples, n, used for the assay has been written in brackets. The standard error (SE) was calculated as the standard deviation divided by the square root of n, where n is the number of assays.

STRAINS	Gene Conversion Rates Mutation rate ± SE(n)	Relative Rate
WTSAY45	$2.8 \times 10^{-6} \pm 1.5 \times 10^{-6}$ (13)	1
ku80Δ	$2.3 \times 10^{-5} \pm 9.5 \times 10^{-6}$ (15)	~8
lig4Δ	$3.5 \times 10^{-5} \pm 6.0 \times 10^{-6}$ (10)	~13
rad50Δ	$6.5 \times 10^{-4} \pm 8.8 \times 10^{-5}$ (18)	~230
mre11Δ	$2.8 \times 10^{-3} \pm 1.1 \times 10^{-3}$ (15)	~1000

FIGURES

Figure 4-1.

Telomere length in strains compromised for NHEJ.

DNA blot of *Eco*R1 digested chromosomal DNA hybridized with a probe specific to *K. lactis* telomeric repeats. **a)** Telomere lengths from strains CK214-4C (wt), AKY124 (*lig4*), SAY572 (*nej1*), SAY573 (*ku80*), SAY579 (*mre11*) and SAY557 (*rad50*). **b)** Telomere lengths of strains SAY100 (*sir4*), AKY116 (*sir4 ku80*), SAY703 (*sir4 lig4*), SAY701 (*sir4 nej1*), SAY704 (*sir4 mre11*) and SAY579 (*mre11*). Size markers are shown on the left.

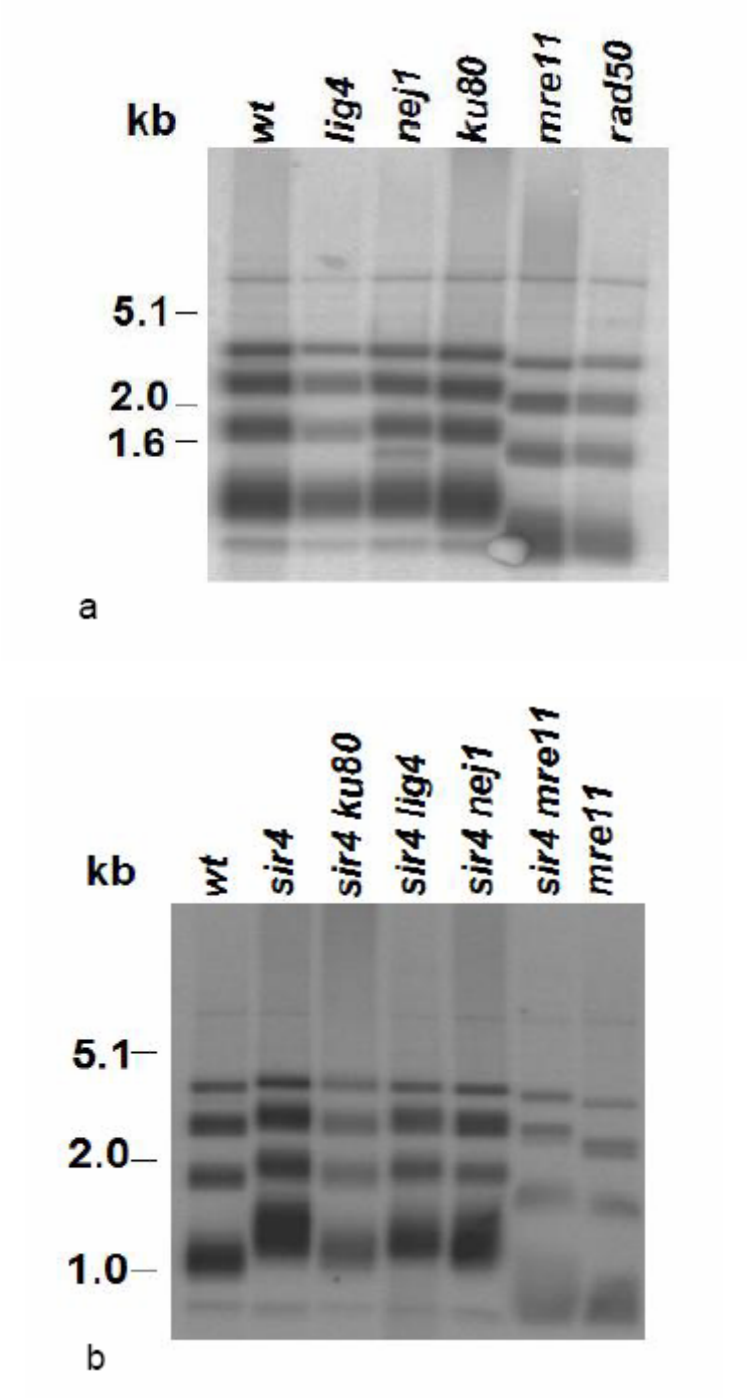

kb
wt
lig4
nej1
ku80
mre11
rad50
5.1
2.0
1.6
a
kb
wt
sir4
sir4 ku80
sir4 lig4
sir4 nej1
sir4 mre11
mre11
5.1
2.0
1.0
b

Figure 4-1

Figure 4-2.

Telomere-telomere fusions (T-TFs) in NHEJ mutant strains.

Size markers shown on the left. **a)** *LIG4*, but not *NEJ1* was required for T-TFs. Shown are two independent isolates of *ter1-4LBsrGI (ter1-4L), ter1-4L lig4* , *ter1-4L nej1* and one *ter1-4L lig4 nej1* strain. Chromosomal DNA was prepared serial re-streaks after generation of the strains. **b**) *KU80* was required for T-TFs. The blot is divided into early and late lanes. Chromosomal DNA from early lanes was isolated immediately following the generation of the mutant strains. Late lanes are of the same mutant strains following 24 streaks. Two independent isolates of *ter1-4L ku80* and one isolate of a *ter1-4L* single mutant are shown. **c**) *ter1-4L mre11* double mutant strains had short, stable telomeres. The blot is divided into early and late lanes as described above. Shown are *ter1-4L, mre11* and *ter1-4L mre11* strains. Wild-types for all blots are TER1 strains recovered from the same tetrads used in the generation of the respective mutant strains. **d**) A *ter1-4L rad 50* double mutant strain incorporated mutant repeats. *Eco*R1 and *Eco*R1/*Bsr*G1 digestions of genomic DNA isolated from a *ter1-4L rad50* mutant strain following 24 serial re-streaks.

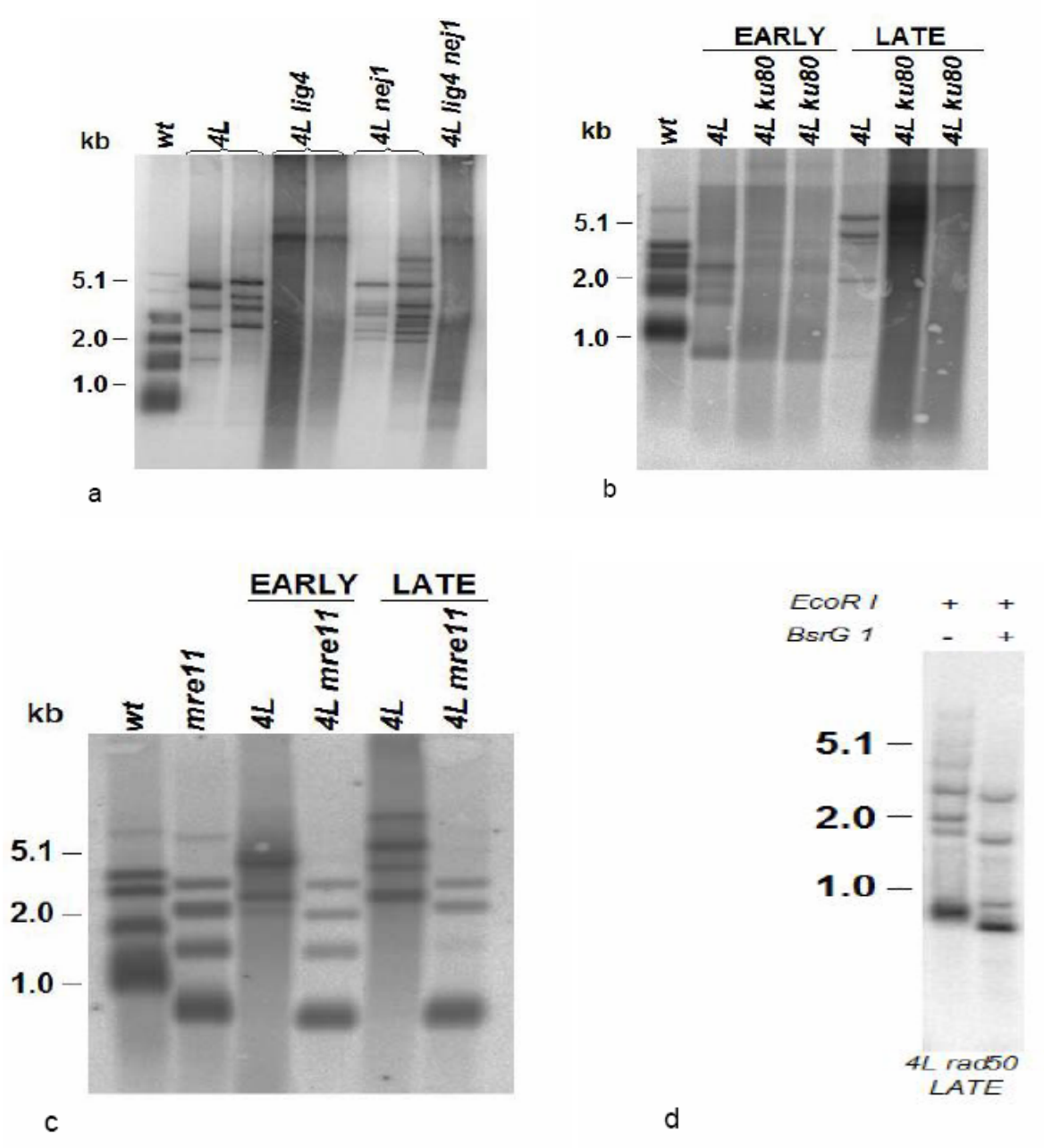
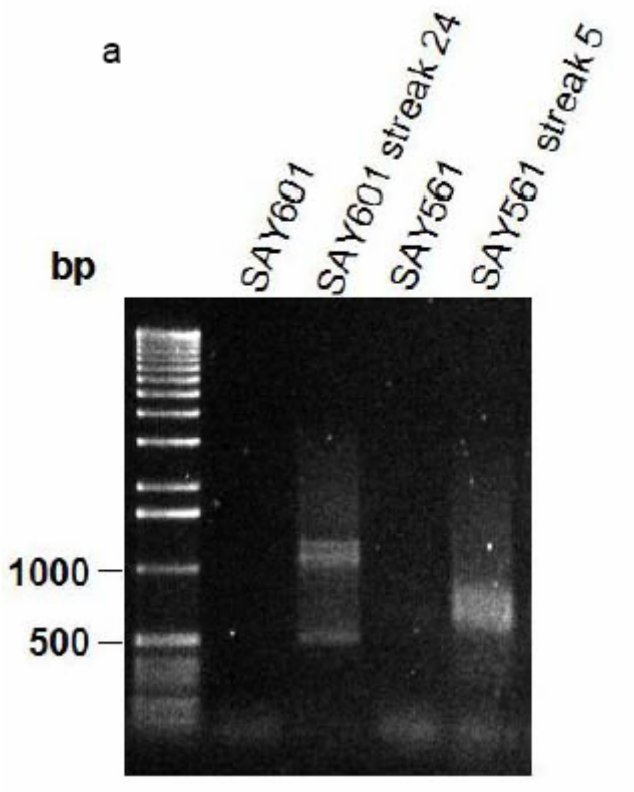

kb
wt
4L
4L lig4
4L nej1
4L lig4 nej1
5.1
2.0
1.0
a
EARLY
LATE
4L ku80
4L ku80
4L ku80
4L ku80
b
mre11
4L mre11
4L mre11
c
EcoR I
BsrG 1
4L rad50
LATE
d

Figure 4-2

Figure 4-3.

Molecular characterization of T-TFs.

a) Agarose gel electrophoresis of PCR amplifications using genomic DNA from pre- and post-fusion strains, as indicated above the lanes, and subtelomeric specific primers. Results were from strains SAY561 and SAY601 following 5 and 24 serial restreaks, respectively. Control lanes result from PCR of genomic DNA isolated from the parental strains used in the generation of each mutant strain prior to the *TER1* loop out procedure. Size markers indicated on the left. **b)** The table shows *K. lactis* T-TFs analyzed by restriction digests and DNA sequencing. PCR amplification of T-TFs using chromosomal DNA from strains that had undergone T-TFs as template, was performed. The resulting DNA fragments were cloned and DNA sequenced. Seven different T-TFs (first column) were analyzed, 1 to 3 originates from a strain after 25 serial re-streaks and 4 to 7 from a strain after 5 serial re-streaks. The second column shows an estimate of the number of mutant repeats present as determined by *Bsr*G1 digestion of each cloned fragment followed by agarose gel electrophoresis. The third column shows a schematic of the sequencing results. Sequenced wild-type repeats are shown as black arrows. Sequenced mutant repeats are shown as blue arrows. Red and yellow boxes represent subtelomeric sequences. The numbers within brackets indicate the estimated difference, in base-pairs, between the fragment submitted for sequencing and the actual sequence recovered from the analysis.



b

Telomere fusion #	Estimated # of mutant repeats BsrG1 digest	Sequenced repeats (wt / mutant)
1	1	[31bp]
2	1	[24bp]
3	1	[-5bp]
4	4	[18bp]
5	19	[279bp]
6	2	[71bp]
7	10	[6bp]

Figure 4-3

Figure 4-4.

Long 3' overhangs in *ku80* cells.

Shown is a non-denaturing in-gel hybridization of genomic DNA of WT, *ku80*, *mre11* and *rad50* strains. They have been digested with three different enzymes *Pst*I, *Msp*I and *Pvu*II (empty square, black oval and grey square) respectively. The left half of the gel has been hybridized to a G-strand telomeric probe and the right half shows the same set of samples probed with a C-strand telomeric probe. Molecular weight markers (M) are in Kb.

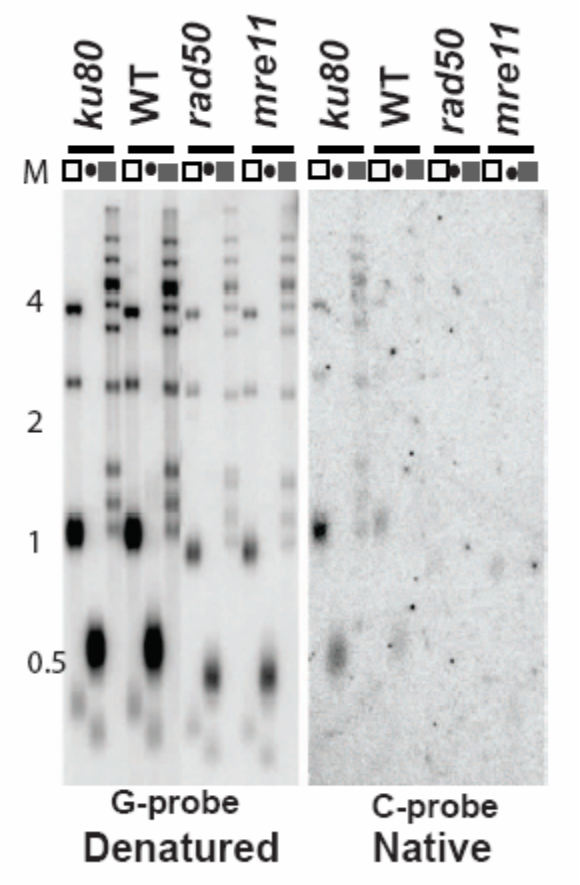

ku80
WT
rad50
mre11
ku80
WT
rad50
mre11
M
4
2
1
0.5
G-probe
Denatured
C-probe
Native

Figure 4-4

Figure 4-5.

Multiple T-TFs are detrimental to *ter1-AccSna* strain undergoing meiosis

Shown is a Southern blot of an *Eco* RI digested genomic DNA of several spores marked 1-16 of a *ter1-AccSna* strain known to form stable fusions. Lanes marked as 7B520, GG1958, *ter1-AccSna* represent the parental haploid controls used to set up the crosses. The presence of sharp bands in the individual spore's lanes, indicates an unaltered fused chromosome, which has undergone meiosis and retained telomere sequences similar to the haploid parent. The genomic DNA was hybridized to a G-strand telomere probe (Klac1-25). Molecular weight markers (M) are in Kb.

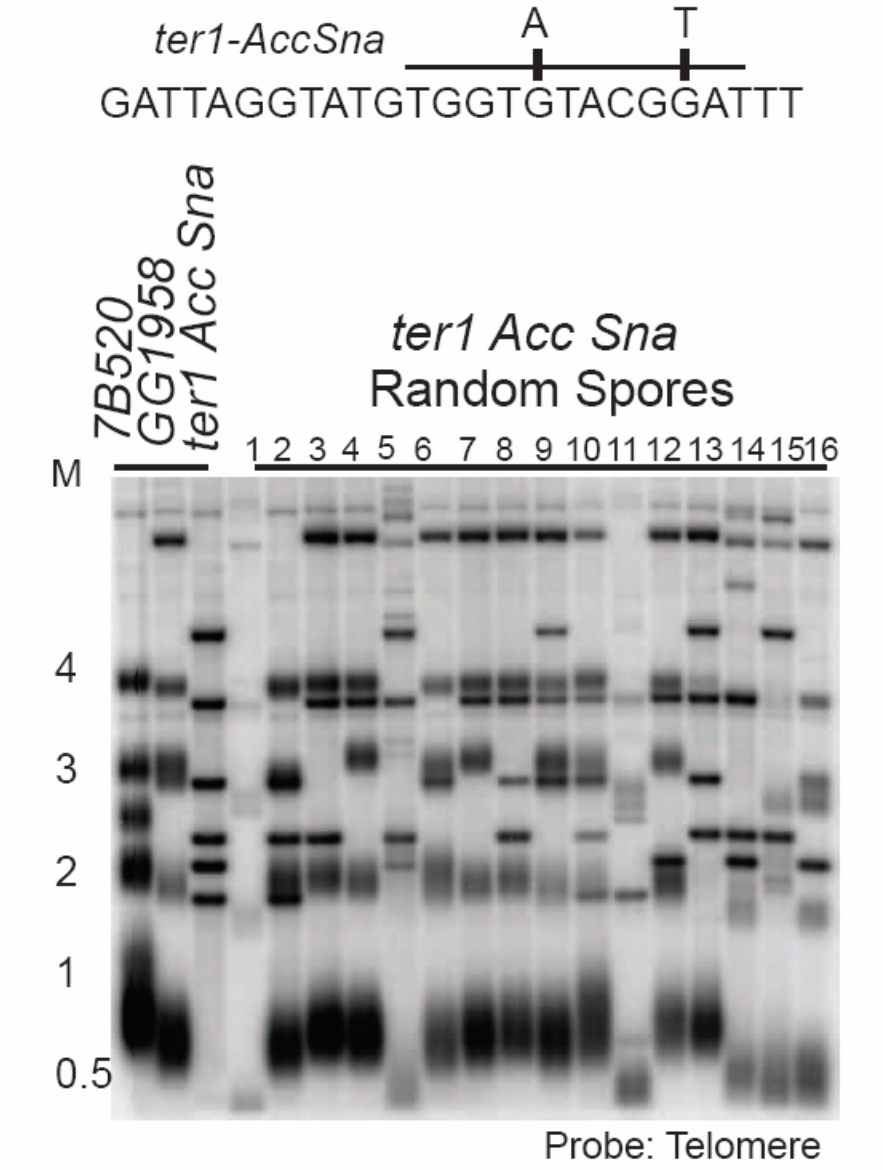

ter1-AccSna
A
T
GATTAGGTATGTGGTGTACGGATTT
7B520
GG1958
ter1 Acc Sna
ter1 Acc Sna
Random Spores
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
M
4
3
2
1
0.5
Probe: Telomere

Figure 4-5

CHAPTER 5

CONCLUSIONS AND FINAL THOUGHTS

Although I have always been very interested in all aspects of telomere biology, the most fascinating concept that has captured my attention is the fundamental difference between end protection and end replication of chromosomes in different organisms. The other vital concept that has always intrigued me is how these two functions provide a basis for understanding disease processes affecting human health and longevity. This dissertation has focused on the consequences of the loss of this protective function, with the hope that the results would provide clues to separate the two functions of *continued prevention* of detrimental changes, and *preservation* of the original form. These results are expected to broaden our overall understanding of how telomeres protect chromosomes and provide genome stability in various organisms.

Genomic instability and Cancer

For a cell in any organism, preserving the integrity of its genome is very vital to ensure its survival. In eukaryotes,if the cell fails to perform this function, and continues on with cell division, this would lead to gross chromosomal aberrations, loss of end protection and eventual demise of the organism. The fact that cancer is a result of increased genomic instability has sparked a flurry of research in recent times. The general understanding is that most cancers arise due to the accumulation of several gene mutations, which in turn lead to telomere dysfunction. Telomere shortening selects for immortalization by engagement of either ALT or telomerase pathways for telomere length regulation (5, 7). However, very little is known about the molecular mechanisms that create this instability and how gross chromosomal abnormalities stemming from dysfunctional telomeres are suppressed in normal cells. I postulate that in cancer

cells, a compromised telomere cap might be one of the driving forces behind the deregulation of telomere length, as observed in early tumorigenesis. Since several human genes involved in telomere function appear to have yeast homologues or equivalents [reviewed in (9)], we proposed that yeast mutants displaying telomere-capping defects would shed light on the mechanisms that go awry in some cancer cells.

Could the immortality of certain cancers arise due to telomere capping defects?

Towards these goals, we first isolated and characterized a gene mutation (*stn1-M1*) in the critical telomere capping protein Stn1, in the yeast *K. lactis*. This mutation led to abnormal growth morphology similar to senescing yeast cells lacking telomerase and very long and heterogeneous telomeres that were maintained by recombination despite an active telomerase regulation pathway. We also observed an excess of single-stranded telomeric DNA, elevated subtelomeric recombination and the formation of a wide range of telomeric circles. Our results also show that *stn1-M1* cells undergo a very high rate of telomere rapid deletion (TRD) upon reintroduction of WT *STN1*, consistent with the mutant having telomere capping defects (Chapters 2 & 3 and the references within). A striking observation was the similarities in the phenotypes observed between *stn1-M1* cells and tumor cells immortalized by activating the ALT pathway involving recombination (1, 8, 10). Overall, our data suggest that both ALT and *stn1-M1* cells share many properties resembling chronic capping defects and are frequently engaged in recombination repair activities as illustrated in Figure 5-1, F and G. However, the mechanisms triggering such loss of the protective function and telomere dysfunction in ALT and *stn1-M1*- cells still remain to be determined.

How can this work refine the bigger emerging picture?

An obvious future course of research suggested by our work is that the phenotype of ALT cells could arise from mutations in genes encoding proteins that are components of the single-stranded-DNA telomere-binding complex. It would therefore be worthwhile to conduct experiments for identifying genes activating the ALT pathway. A second exciting possibility that stem from our work is the importance of recombination genes necessary for contributing to the phenotype in ALT or *stn1-M1* cells. A better understanding of the genes regulating the type IIR pathway in *stn1-M1* cells could lead to therapeutics designed specifically to treat ALT tumors because of our result with *stn1-M1* cells, which failed to survive in the absence of *RAD52* gene.

In addition, a number of fundamental and very interesting questions on the possible mechanism (s) of regulating end protection can be answered by conducting further studies in the *stn1-M1* mutant in *K. lactis.* For instance, how does Stn1 protect telomeres in normal WT yeast cells? Does it aid in forming a protective structure (Figure 5-1 A-D) and how does the *stn1-M1* mutation disrupt it? Does Stn1p have structural oligosaccharide binding folds (OB) and bear structural similarity to the Oxytricha β subunit of the telomere end binding protein (TEBP) (4)? An abundance of t-circles seem to be a hallmark of capping defect in *stn1-M1* cells and in various organisms elsewhere (Chapter 3 and references within). What does it tell us about the role of the circles in the *stn1-M1* cells? Could they arise as a consequence of telomere rapid deletions? What is the mechanism of telomere rapid deletions in *stn1-M1* cells? Could circles be the driving force by forming templates in rolling circle replication events leading to runaway RTE? What is the role of the circles in various classes of mutants (RNA template mutants) not requiring recombination to elongate telomeres in *K. lactis?* What are the genes regulating these pathways?

Possible experiments to address some of these questions are discussed below.

1. Preliminary experiments in *K. lactis* have indicated that telomeres in wild type cells lack t-loops and are presumed to be protein bound at the end (Figure 5-1A). However, they can form protected t- loop structures (Figure 5-1 C) in certain mutants (Cesare et. al in prep). Some evidence in *S. cerevsiae* has also indicated that telomeres form fold-back structures brought about by the binding of Rap1 (Figure 5-1 D). However, data remains inconclusive at this point, because of the challenges one has to undergo in preserving such transient structures for electron microscopy. Nevertheless, if one overcomes these technical difficulties, it will be worthwhile to conduct similar experiments in WT cells of *S. cerevisiae* so that one will be able to draw comparisons between the end structures in the two yeast strains.
2. Overall, our data suggest that Stn1 protects telomeres against initiating homologous recombination events in normal WT cells. This protection might occur directly through the interaction of the Stn1/Cdc13/Ten1 complex with telomeric DNA (2). To test this, one can design experiments to verify if the binding of Cdc13 to telomeric DNA is disrupted in the *stn1-M1* cells in *K. lactis*.
3. Alternately, Stn1 might indirectly provide end protection by interacting with the components of DNA polymerase α/primase to regulate synthesis of the C-rich strand at 3' G-strand telomeric overhangs (3). One way to study it is to disrupt DNA polymerase components in the *stn1-M1* background and study the consequences of it. However, this returns to the fundamental question raised in the beginning of this chapter as to whether one can really distinguish between end protection and end regulation.

All mixed up in the end

It is widely accepted that telomeres with a compromised cap can engage in both homologous recombination (HR) and nonhomologous end joining (NHEJ) (Figure 5-1 E, F). HR-repair can result in telomerase independent telomere elongation or telomere shortening. Recent data appears to be a little confusing because of the overlapping proteins involved in performing both these functions (6). To address this question, we examined the role of several *K. lactis* genes in the regulation of telomere length and the ability of telomeres to protect against homologous recombination and non-homologous end joining (Chapter 4). We conclude that NHEJ proteins can have multiple roles at *K. lactis* telomeres including mediating fusion leading to circularization of long mutant telomeres (Figure 5-1) and ensuring end protection of wild type telomeres.

In conclusion, one could ask if chromosome circularization be considered a result of a compromised capped state or if the organism evolved into a more sophisticated capped state by losing all its telomeres and by forming circular chromosomes. Most likely our next step in understanding such a complexity will be to understand the basic requirement of telomere DNA sequences for performing the telomere function. We hope this will provide further clues to a much deeper understanding of the origin of telomere capping and maintenance systems in eukaryotes.

REFERENCES

1. **Cesare, A. J., and J. D. Griffith.** 2004. Telomeric DNA in ALT cells is characterized by free telomeric circles and heterogeneous t-loops. Mol Cell Biol **24:**9948-57.

2. **Evans, S. K., and V. Lundblad.** 2000. Positive and negative regulation of telomerase access to the telomere. J Cell Sci **113 Pt 19:**3357-64.

3. **Grossi, S., A. Puglisi, P. V. Dmitriev, M. Lopes, and D. Shore.** 2004. Pol12, the B subunit of DNA polymerase alpha, functions in both telomere capping and length regulation. Genes Dev **18:**992-1006.

4. **Horvath, M. P., and S. C. Schultz.** 2001. DNA G-quartets in a 1.86 A resolution structure of an Oxytricha nova telomeric protein-DNA complex. J Mol Biol **310:**367-77.

5. **Kim, N. W., M. A. Piatyszek, K. R. Prowse, C. B. Harley, M. D. West, P. L. Ho, G. M. Coviello, W. E. Wright, S. L. Weinrich, and J. W. Shay.** 1994. Specific association of human telomerase activity with immortal cells and cancer. Science **266:**2011-5.

6. **Louis, E. J., and A. V. Vershinin.** 2005. Chromosome ends: different sequences may provide conserved functions. Bioessays **27:**685-97.

7. **Murnane, J. P., L. Sabatier, B. A. Marder, and W. F. Morgan.** 1994. Telomere dynamics in an immortal human cell line. Embo J **13:**4953-62.

8. **Perrem, K., T. M. Bryan, A. Englezou, T. Hackl, E. L. Moy, and R. R. Reddel.** 1999. Repression of an alternative mechanism for lengthening of telomeres in somatic cell hybrids. Oncogene **18:**3383-90.

9. **Rouse, J., and S. P. Jackson.** 2002. Interfaces between the detection, signaling, and repair of DNA damage. Science **297:**547-51.

10. **Wang, R. C., A. Smogorzewska, and T. de Lange.** 2004. Homologous recombination generates T-loop-sized deletions at human telomeres. Cell **119:**355-68.

FIGURES

Figure 5-1.

Protective structures in different organisms.

(A) Terminal proteins covalently attached to the 5’ end. Examples of such structures have been observed in adenovirus, streptomyces and several mitochondria and fungal species. Protein bound is shown in black. (**B**) Terminal hairpins/palindrome sequences. Examples of such structures have been observed in linear chromosomes of *Borrelia*, poxvirus, mitochondria of yeasts and protozoa. (**C**) Telomeric loops (t-loops). Examples of such structures have been observed in mammals, mitochondria of fungi, plants. (**D**) Terminal fold back structure observed in budding yeast *S. cerevisiae.* This structure is brought about by binding of Rap1 (grey ovals) which leads to bending of DNA.

Consequences of loss of end-protection

(**E**) NHEJ-mediated fusions. Circularization of linear chromosomes due to a compromised cap. Telomeric repeats are shown in black dots and chromosomes are in grey. (**F**) HR-mediated telomere elongation and deletion. The left panel indicates lengthening of critically short telomeres by rolling circle DNA synthesis. The right panel is intrachromosomal deletion events leading to truncation of telomere repeats. (**G**) Loss of 5’ telomeric DNA sequence by nucleases leading to long 3’ overhangs [Reviewed in (6) and references within].

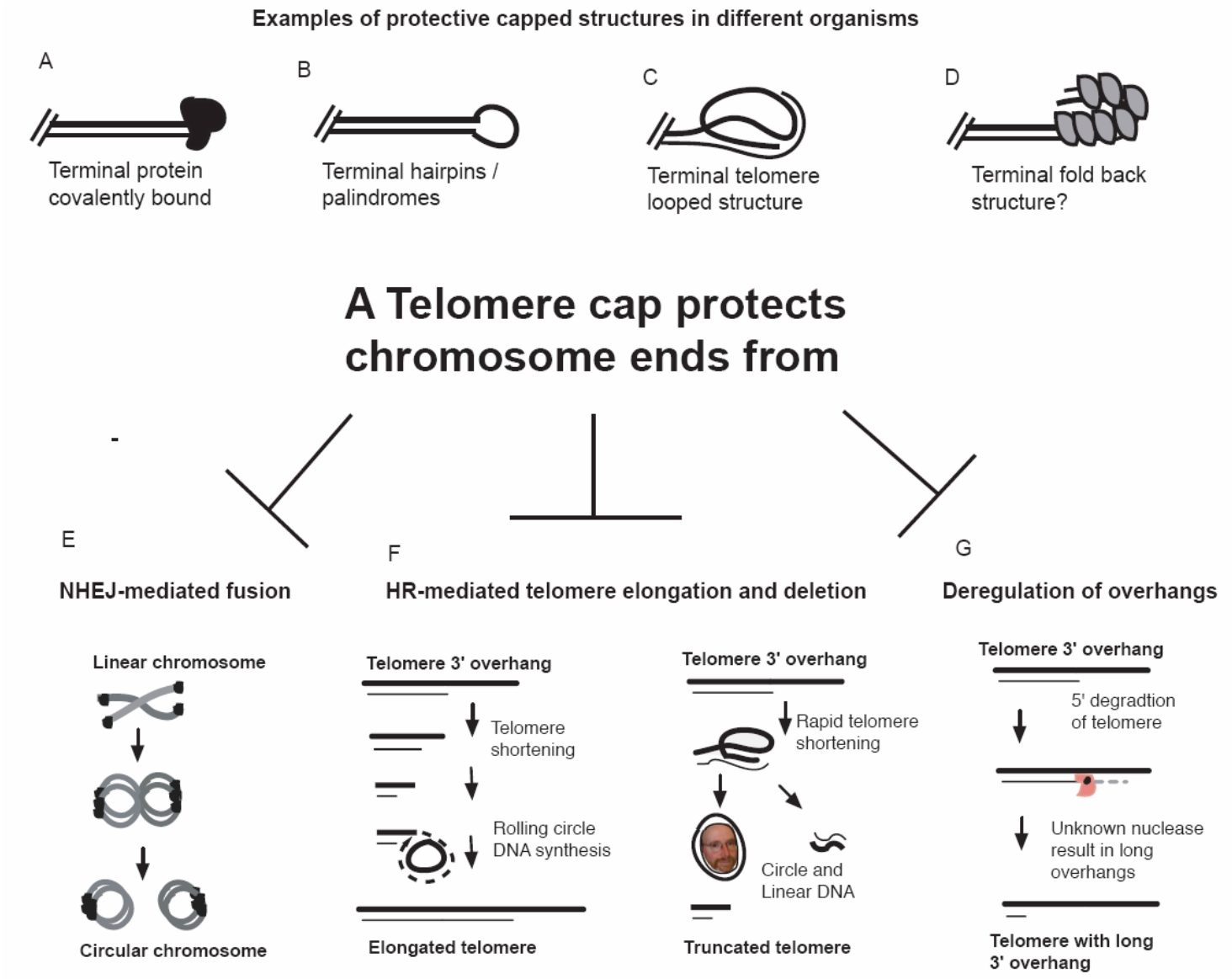

Examples of protective capped structures in different organisms
A
Terminal protein covalently bound
B
Terminal hairpins / palindromes
C
Terminal telomere looped structure
D
Terminal fold back structure?
A Telomere cap protects chromosome ends from
E
NHEJ-mediated fusion
F
HR-mediated telomere elongation and deletion
G
Deregulation of overhangs
Linear chromosome
Circular chromosome
Telomere 3' overhang
Telomere shortening
Rolling circle DNA synthesis
Elongated telomere
Telomere 3' overhang
Rapid telomere shortening
Circle and Linear DNA
Truncated telomere
Telomere 3' overhang
5' degradtion of telomere
Unknown nuclease result in long overhangs
Telomere with long 3' overhang

Figure 5-1

APPENDIX

ODE TO TELOMERES

Telomerase

"On the night of Christmas Eve in 1984,
Carol and Liz were working hard just like before,
And Carol streamlined the enzyme activity with grace,
While Liz named it Telomere Terminal Transferase.
Together they published about 'TTT' in Cell and Nature,
For in those good old days, it was such an easy feature.
This enzyme renamed as Telomerase,
Has been classified as a reverse transcriptase,
For it uses its RNA subunit as a template,
To add telomere repeats at the ends at an even pace.
The popularity of Telomerase has grown in recent years,
For this very enzyme immortalizes almost all tumors.

RTE

An evening in the lab with just Kluyveri and Me,
Mike chimed out the abbreviation -R-T-E!
"What does it mean?" I turned around and 'quipped;
Recombinational Telomere Elongation, "duh uh", he chipped.
"Recombinatio*al*?" I frowned, I have never heard of that before,
Instantly Mike replied, "it has been cited a dozen times or more!"
Pray tell me dear Kluyveri, do you like this name for thee?
'Of course' replied she, for it seems to fit quite nicely.
Therefore grudgingly I accepted, and RTE stayed as it is,
On the two types of pathways loved by Mike, Kluveri and me;

My budding friend Kluyveromyces

The two pathways Type II and Type II R RTE
Quietly reside in my dear old Kluyveri.
Alas, they can get wild by recombination, you see
When telomeres lose their caps occasionally.
And yet they can be controlled quite nicely;
When recapped, by telomerase or Stn respectively.
And yet the two pathways in dear old kluyveri,
Have always been best buddies to me.

Rolling Telomeres

In ‘97, I interviewed for a faculty named Michael,
Who had a shy smile, yet his eyes had a sparkle;
And in his lab I learnt new scientific tricks,
And his discovery of 25 base pair, repeats in K. lactis.
In this way we published two main papers,
On recombination and fusion,
Of Kluyveromyces telomeres.
And I moved on to characterize a ‘capping’ gene mutation,
And he the model for ‘roll and spread’ recombination.
I will cherish those loving and formative years to the very end;
For Michael and Kluyveri, are two of my very best friends.